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CONTENTS

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Additions and Corrections

Vol. 20, No. 4

page	column	line	for	read
165	FIG. 1.		–•–•–•–	·····
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174	right	18	Mcilvaine's	McIlvaine's
177	left	24	acetic anhydride (12 ml)	acetic anhydride (12 ml) and 2 N NaOH (125 ml)

Vol. 20, Suppl.

257	FIG. 1.		Adsorption	Absorption
258	left	1, 3	adsorption	absorption

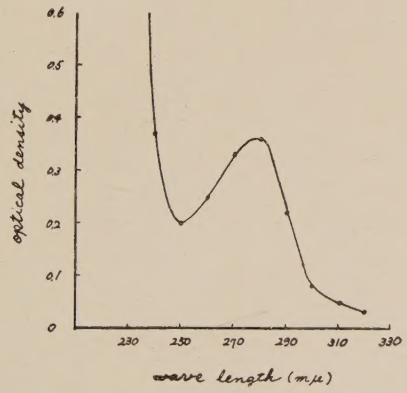
Vol. 21, No. 1

71	right	19	recovered,	recovered, as

Corrected Figures

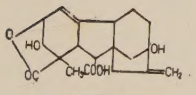
Vol. 20, Suppl.

page 257 FIG. 1



Vol. 21, No. 1

page 74 (IV)



(IV)

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 159~164, 1956]

Biochemical Studies on "Bakanae" Fungus. Part XXXVI
The Physiological Action of Gibberellin. VIII

Changes in the Activities of Various Enzymes in Leaf-Sheaths of Rice Plants Treated with Gibberellin

By Takeshi HAYASHI, Yutaka MURAKAMI and Shôichi MATSUNAKA

Section of Physiology and Genetics, National Institute of Agricultural Sciences.

Received May 21, 1956

The seventh leaf-sheaths of rice plants grown in solution and treated with gibberellin were analyzed to examine their changes in the activities of various enzymes during period of growth, in comparison with that of the control plants. Phosphatase, alkalipyrophosphatase, acetylesterase, maltase, β-glucosidase, α-galactosidase, β-galactosidase, amylase, urease, dipeptidase, ascorbic acid oxidase, and catalase activities were decreased in extracts of sheaths on a fresh weight basis by treatment with gibberellin. Activities of peroxidase and invertase were markedly increased.

It has been known that growing leaf-sheaths begin to increase in length when rice plants are cultured in a definite concentration of gibberellin (GIB). In an effort to gain some clue to the mode of the action of GIB, the writers have reported the chemical changes concomitant with the application of this substance to rice plants and the differences in the mode of action between GIB and auxins by the various growth tests.[1,2,3,4] It has been shown that the growth of the cell in intact organs may be accompanied by a considerable increase in the protein content.[5] This suggests that in the conditions in which the increase occurs there may be corresponding changes in the activites of various enzymes. Therefore, it is of interest to estimate changes in the enzyme pattern of sheaths of GIB-treated rice plants with that of control plants. The following enzymes were selected for study in the present work; phosphatase, alkali-pyrophosphatase, acetylesterase, maltase, β-glucosidase, α-galactosidase, β-galactosidase, invertase, amylase, urease, dipeptidase, ascorbic acid oxidase, peroxidase, and catalase. The choice of these enzymes was not determined by any assumptions regarding their importance for the process of growth, but by the relative ease of estimating the reactions which are promoted by them.

1) T. Hayashi, Y. Takijima, and Y. Murakami, *J. Agr. Chem. Soc. Japan*, **27**, 672 (1953).
2) T. Hayashi and Y. Murakami, *ibid*, **27**, 675 (1953).
3) T. Hayashi and Y. Murakami, *ibid*, **27**, 797 (1953).
4) T. Hayashi and Y. Murakami, *ibid*, **28**, 543 (1954).
5) R. Brown and D. Broadbent, *J. Exp. Bot*, **1**, 247 (1951).

MATERIAL AND METHODS

Rice plants (Aichi-Asahi), which were grown in the seed bed in summer, were selected for transferring to Kasugai solutions when they were about 20 cm in height with five developed leaves. The culture vessels were 500 ml wide-mouth glass jars, each containing five rice plants. They were placed in the glasshouse. When the unfolded seventh leaf had appeared about 5 cm above the sixth leaf, the plant was separated into two groups at random. One group was supplied with crude GIB at a concentration of 10 mg/l. Plants were harvested immediately after the treatment. A similar number of control and treated plants had been harvested three times after GIB introduction before the seventh

sheaths had almost completed their growth. Harvestings were made from 10 to 12 a.m. Under the experimental conditions chosen, no extension growth of sheaths below the sixth sheath took place after treatment of the plants.

The following procedure was employed in preparing the enzyme solution. Excised sheaths were thoroughly ground in mortars with 5.0 ml of distilled water and 1.0 gm of silica sand per gram of tissue and the resulting homogenates were then centrifuged at 1,000 r.p.m. for 2 minutes. Supernatants from this centrifugation were used as the enzyme solution and the content of total-N was then determined by the micro-Kjeldahl procedure. They were used without further dilution for the estimation of alkalipyrophosphatase, α-galactosidase, amylase, urease, dipeptidase, ascorbic acid oxidase, and catalase activities. They were diluted 1:2 for the assay of phosphatase, maltase, β-glucosidase, β-galactosidase, and invertase activities, and were diluted 1:10 and 1:50 for acetylesterase and peroxidase activities, respectively. Unless otherwise noted, the reaction temperature was at 30°C. In preliminary work on methods of assay for the various enzyme activities, it was found that when these assay methods described below were employed, the relationship between reaction rate and enzyme concentration approached linearity over a several-fold range in concentration of the enzyme.

Phosphatase assay.

Reaction mixture :

0.048 M Sodium *p*-nitrophenylphosphate	1.0 ml.
0.5 M Acetate buffer, pH 5.9	1.0 ml.
Distilled water	1.0 ml.
Enzyme solution	1.0 ml.

p-Nitrophenol liberated from a 1 ml aliquot was determined colorimetrically. Enzyme activity was expressed as a velocity constant of the first order reaction (K).

Alkalipyrophosphatase assay.

Reaction mixture :

0.0358 M Sodium pyrophosphate	1.0 ml.
0.2 M Tris buffer, pH 8.7	5.0 ml.
0.12 M $MgCl_2$	1.0 ml.
Enzyme solution	1.0 ml.

Inorganic phosphorus liberated from a 1-ml aliquot was determined by the method of Fiske and Subbarrow. Enzyme activity was expressed as a velocity constant of the first order reaction (K).

Acetylesterase assay.

Reaction mixture :

0.33 mM *p*-Nitrophenylacetate	2.0 ml.
1/15 M Phosphate buffer, pH 7.0	2.0 ml.
Distilled water	5.0 ml.
Enzyme solution	1.0 ml.

Enzyme activity was expressed by an optical extinction reading after 30 minutes, with a photoelectric colorimeter (Shimadzu) at 450 mμ, using a 3.00-cm square cuvette.

Maltase assay.

Reaction mixture :

0.048 M Maltose	1.0 ml.
0.5 M Acetate buffer, pH 5.2	1.0 ml.
Enzyme solution	2.0 ml.

Glucose liberated from a 0.5 ml aliquot was determined reductometrically by the method of Shaffer-Hartmann. Enzyme activity was expressed as a velocity constant of the first order reaction (K).

β-Glucosidase assay.

Reaction mixture :

0.048 M *p*-Nitrophenyl-β-D-glucoside	1.0 ml.
0.5 M Acetate buffer, pH 5.2	1.0 ml.
Enzyme solution	2.0 ml.

p-Nitrophenol liberated from a 0.5 ml aliquot was determined colorimetrically. Enzyme activity was expressed as a velocity constant of the first order reaction (K).

α-Galactosidase assay.

Enzyme activity was estimated under the same condition as for the experiment of maltase except that in this case maltose was replaced by melibiose.

β-Galactosidase assay.

Enzyme activity was estimated under the same condition as for the experiment of β-glucosidase activity except that *p*-nitrophenyl-β-D-galactoside was used as substrate.

Invertase assay.

Reaction mixture :

0.048 M Sucrose	1.0 ml.
0.5 M Acetate buffer, pH 5.2	1.0 ml.
Distilled water	1.0 ml.
Enzyme solution	1.0 ml.

The expression of enzyme activity was the same

as described under the maltase assay.

Amylase assay.

Reaction mixture:

2.5% Soluble starch	2.0 ml.
0.1 M Acetate buffer, pH 4.5	1.0 ml.
Enzyme solution	1.0 ml.

Reducing sugars liberated from a 1.0 ml aliquot were determined reductometrically by the method of Shaffer-Hartmann. Enzyme activity was expressed as a velocity constant of the first order reaction (K).

Urease assay.

Reaction mixture:

1.0 M Urea	1.0 ml.
0.5 M Phosphate buffer, pH 6.5	1.0 ml.
Enzyme solution	2.0 ml.

Ammonia produced from a 0.5 ml aliquot was determined colorimetrically, through the use of Nessler's reagent. Enzyme activity was expressed as a velocity constant of the first order reaction (K).

Dipeptidase assay.

Dipeptidase activity was estimated by the method of Linderström-Lang and Sato[6] using glycylglycine as the substrate, and was expressed as percent hydrolysis after 48 hours.

Ascorbic acid oxidase assay.

Enzyme activity was determined manometrically in a Warburg apparatus. Each reaction vessel contained 1.0 ml of enzyme solution and 0.8 ml of 0.2 M phosphate buffer, pH 6.0. Then 0.3 ml of 20% KOH was added to the center well. The sidearm contained 0.2 ml of 0.1 M ascorbic acid. Activity was expressed in the amount of O_2 uptake during the first hour.

Peroxidase assay.

Reaction mixture:

0.1% Guaiacol	1.0 ml.
0.1% H_2O_2	1.0 ml.
0.5 M Acetate buffer, pH 5.0	1.0 ml.
Enzyme solution	2.0 ml.

At 2, 5, 10, and 15 minutes, 1.0 ml aliquots were pipetted from the reaction mixture into 1.0 ml portions of 1 N H_2SO_4 and water added up to 10.0 ml. The optical extinction was then read with a photoelectric colorimeter at 450 mμ, using a 3.00-cm square cuvette. The activity was expressed as the optical extinction readings after 5 minutes.

Catalase assay.

Catalase activity was estimated by the method of Euler and Josephson[7].

Total-N and protein-N contents of the sheaths were determined by the method of Christiansen and Thimann[8].

RESULTS AND DISCUSSION

The effect of GIB treatment on growth increments of the seventh leaf-sheaths is shown in Fig. 1. The increases in length,

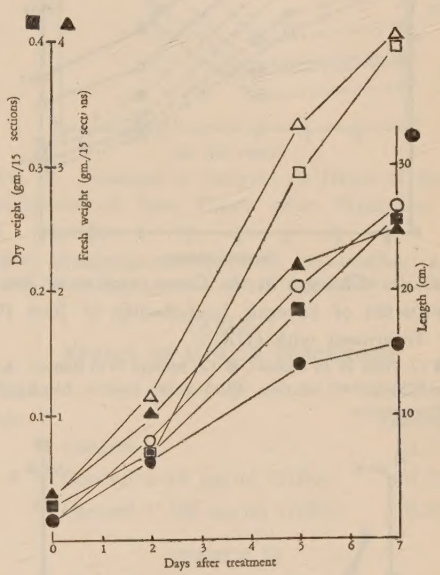


FIG. 1. Growth of Seventh Leaf-sheaths of Rice Plants Treated with GIB Related to That of Untreated Control.

● ○, length; ■ □, dry weight; ▲ △, fresh weight. Black marks, control; blank marks, GIB-treatment.

fresh-weight, and dry-weight of the seventh leaf-sheaths were very remarkable as the result of GIB treatment. On the other hand, GIB treatment reduced both total-N and protein-N in sheaths if the concentration of these constituents was calculated on a dry-weight basis (Fig. 2). As reported in a

6) K. Linderström-Lang and M. Sato, *Z. Physiol. Chem.* **184**, 83 (1929).

7) J. B. Sumner and G. F. Somers, "Laboratory Experiments in Biological Chemistry", Academic press, (1949), p. 147.

8) G. S. Christiansen and K. V. Thimann, *Arch. Biochem.* **26**, 230 (1950).

previous paper[1], these facts suggest that this increase in dry weight is largely due to an increase in cell-wall materials, such as cellulose.

Enzyme solutions, obtained by the procedure mentioned above, were analyzed for total-N, and various enzymes. The results are represented graphically in Figs. 2–7. Enzyme solutions from GIB treated sheaths showed somewhat lower content of nitrogen and also lower activities in almost all the estimated enzymes. It is significant, however, that the activities of peroxidase and invertase increased as a result of GIB treatment despite the above fact that the total-N decreased.

GIB-A[9] was added to the enzyme solution, which was obtained from rice plant leaves by the tannic acid precipitation method[10], in order to examine whether GIB directly increased the activities of the above two enzymes. The results of Tables I. and II. showed no enhancement of activities by GIB

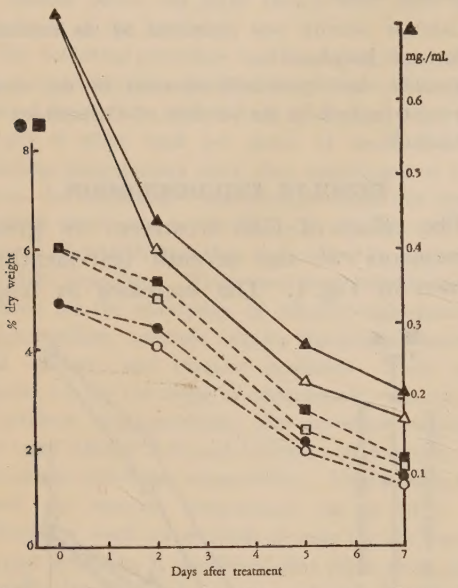


FIG. 2. Changes in the Concentration of Nitrogen Constituents of Seventh Leaf-sheaths of Rice Plants after Treatment with GIB.

■ □, total N in tissues; ● ○, protein N in tissues: ▲ △, total N in enzyme solution. Black marks, control; blank marks, GIB-treatment.

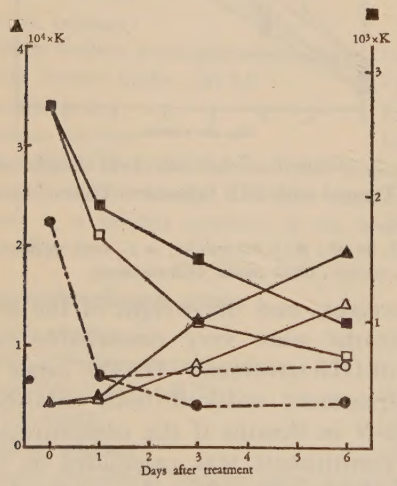


FIG. 3. Changes in Enzyme Activities of Seventh Leaf-sheaths of Rice Plants after Treatment with GIB. (Part 1).

■ □, phosphatase; ● ○, invertase; ▲ △, amylase. Black marks, control; blank marks, GIB-treatment.

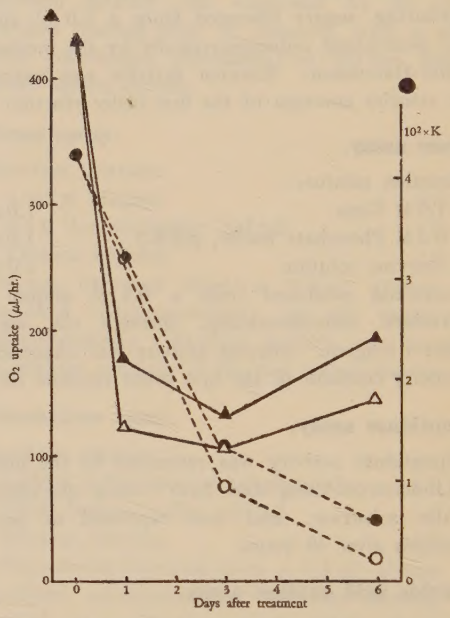


FIG 4. Changes in Enzyme Activities of Seventh Leaf-sheaths of Rice Plants after Treatment with GIB. (Part 2).

● ○, catalase; ▲ △, ascorbic acid oxidase. Black marks, control; blank marks, GIB-treatment.

9) N. Takahashi et al., This Bulletin, **19**, 267 (1955).
10) K. Niwa, *J. Biochem. Japan*, **37**, 301 (1950).

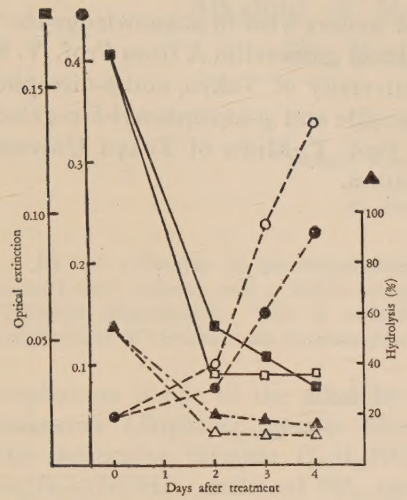


FIG. 5. Changes in Enzyme Activities of Seventh Leaf-sheaths of Rice Plants after Treatment with GIB. (Part 3).

■ □, acetylesterase; ● ○, peroxidase; ▲ △, peptidase. Black marks, control; blank marks, GIB-treatment.

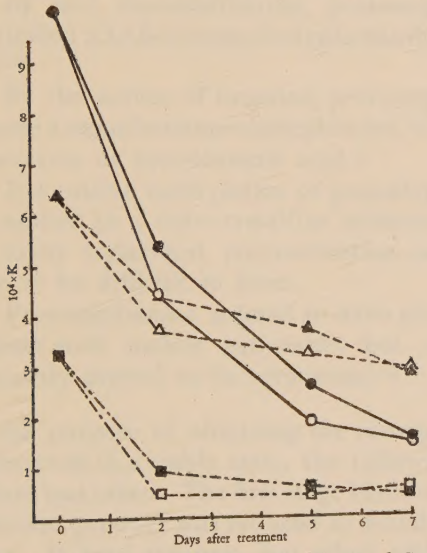


FIG. 6. Changes in Enzyme Activities of Seventh Leaf-sheaths of Rice Plants after Treatment with GIB. (Part 4).

■ □, maltase; ● ○, β-glucosidase; ▲ △, β-galactosidase. Black marks, control; blank marks, GIB-treatment.

added in vitro. This will indicate that the observed increases in peroxidase and invertase activities are the expression of

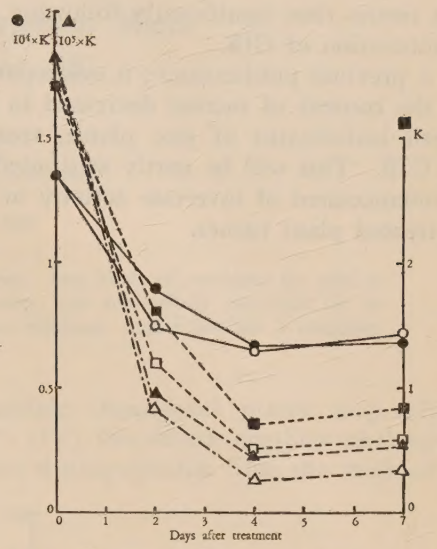


FIG. 7. Changes in Enzyme Activities of Seventh Leaf-sheaths of Rice Plants after Treatment with GIB. (Part 5).

■ □, alkalipyrophosphatase; ● ○, α-galactosidase; ▲ △, urease. Black marks, control; blank marks, GIB-treatment.

TABLE I

EFFECT OF GIB ON PEROXIDASE

Time min.		Extinction readings
5	Control	0.21
	Control + 10 μg/ml GIB-A	0.21
	Control + 100 μg/ml GIB-A	0.21

TABLE II

EFFECT OF GIB ON INVERTASE

Time min.		Hydrolysis %
990	Control	14.2
	Control + 10 μg/ml GIB-A	14.2
	Control + 100 μg/ml GIB-A	13.6

changes in the structure of the protein complex of the cell. Recent evidence[11] has indicated that the oxidation of indoleacetic acid (IAA) in rice plants is due to peroxidase. Since the elongation of plant cells is known to be hormonally regulated by IAA, it is very interesting that peroxidase activity of rice

11) Y. Murakami and T. Hayashi, *J. Agr. Chem. Soc. Japan*, **30**, 414 (1956).

plant tissues rises significantly following the administration of GIB.

In a previous publication[1], it was reported that the content of sucrose decreased in the seventh leaf-sheaths of rice plants, treated with GIB. This will be partly attributed to the enhancement of invertase activity in the GIB-treated plant tissues.

The writers wish to acknowledge the gift of samples of gibberellin A from Prof. Y. Sumiki of University of Tokyo, and *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-galactoside from Prof. T. Miwa of Tokyo University of Education.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 165~173, 1956]

Alkaloid of *Stephania japonica* Miers On Protostephanine (12)

By Kyoji Takeda

Research Foundation ITSUU Laboratory

Received May 23, 1956

By the reduction of protostephanine methylmethine, two kinds of methines (α- and β-isomer) were isolated, and a stable nitrogen-free substance was successfully obtained by its Hofmann degradation. This in connection with other evidence, makes possible a complete assignement of structure for protostephanine.

Protostephanine is one of the alkaloids of Hasunohakazura (*Stephania japonica* Miers). It has the molecular formula $C_{21}H_{27}NO_4 = C_{16}H_{12}(OCH_3)_4(NCH_3)$, melts at 95°, and is optically inactive.

H. Kondo and T. Watanabe, and K. Takeda (under Kondo) have studied this base and established the following facts.

(1) By zinc dust-distillation, protostephanine yielded 2,3,6,8-tetramethoxyphenanthrene (I).[3,4]

(2) By the action of bromine, protostephanine gave a monobromoprotostephanine, with the liberation of hydrobromic acid.[5]

(3) Exhaustive methylation of protostephanine resulted in a non-crystalline substance, which easily underwent polymerisation, and become to be difficult to treat.

(4) Protostephanine seemed to have given an adduct with maleic anhydride, but this was recently proved to be erroneous.[1,6]

For the purpose of obtaining the nitrogen-free substance in a stable state, the following procedure was taken. The first stage Hofmann degradation product was reduced to dihydromethine. It was syruppy, but when added with methyliodide, it gave a crystalline methiodide (hexagonal plates, m. p. 177.5–178.5°) (IV), this dihydromethine methiodide is easily distinguishable from the methiodide

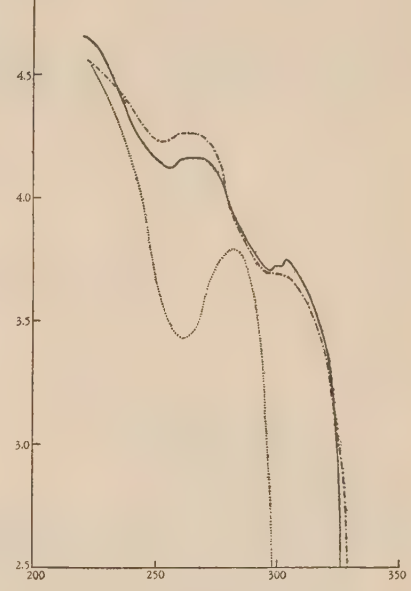


FIG. 1

——— Protostephanine α-methylmethine methiodide (II);
–o–o–o– ,, α-dihydromethine methiodide (IV);
····· ,, β-dihydro-N free substance (VII).

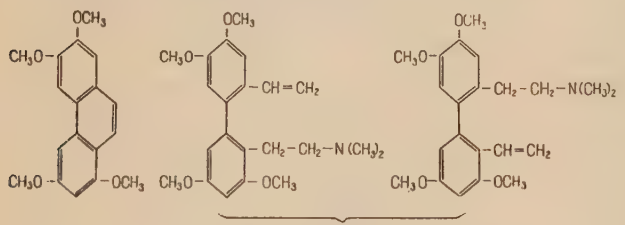
of the original, unreduced methine (rhombic prisms, m. p. 187–188°) (II)[7] in crystal form, in melting form and in ultraviolet absorbtion curve (Fig. 1), although the mixed melting

1) On protostephanine (11). *Ann. Rpt. ITSUU Lab.*, **7**, 71 (1956).
2) Konnocho Shibuya-ku, Tokyo.
3) H. Kondo and T. Watanabe, *Ann. Rpt. ITSUU Lab.*, **1**, 37 (1950).
4) H. Kondo and K. Takeda, *ibid.*, **4**, 51 (1953).
5) H. Kondo, T. Watanabe and K. Takeda, *ibid.*, **3**, 45 (1952).
6) H. Kondo and T. Watanabe, *ibid.*, **1**, 50 (1950).
7) H. Kondo and T. Watanabe (*J. Pharm. Soc. Japan*, **58**, 268 (1938)) reported m.p. 185°.

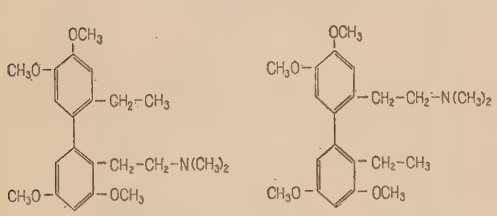
point lies between each of them.

The mother liquor of the above dihydromethine methiodide (IV) gave on standing, however, another methiodide in a small amount, which crystallized in rhombic prisms and melted at 144–146°. By elemental analysis, it was proved that the both methiodide were isomeric.

It became, therefore, to be very suspectable that the dihydromethine, as well as the original unreduced methine (II) must have consisted of two isomers, produced perhaps by the different fission points in the course of the first stage of Hofmann degradation (see the formula II and III).



(I) α- and β-Methine.
Methiodide: (II) and (III).



α- and β-Dihydro-methylmethine
Methiodide: (IV) and (V)

The author wishes for convenience's sake, to name the dihydromethine, whose methiodide melts at 178.5° (IV) as α- and the other, whose methiodide melts at 146° (V) as β-. The same nomenclature can be applied to the original unreduced methines, as from the ratio of yield in the following experiments is shown.

To separate the original, unhydrogenated methine into α- and β- component, the methine was transformed into the picrate. The first crops (m. p. 168°) was set free from picric acid and converted into its methiodide, which melted at 187–188°.

The mother liquor of the above picrate remained syrupy, but the base liberated therefrom gave a methine in a scanty yield, whose methiodide melted at 175–177°.

α-Dihydromethylmethine methiodide (IV) when subjected to the second stage Hofmann degradation gave α-dihydro-N-free substance (VI) as rhombic prisms of m. p. 103.5–104°, which was quite stable and showed no tendency to polymerize. The yield was quantitative.

Reduction of α-dihydro-nitrogen-free substance (VI) with palladiumcharcoal catalyst resulted in the formation of a tetrahydrosubstance (VIII) as rhombic prisms of m.p. 59–60° by the absorption of one mole of hydrogen (Fig. 2).

The Hofmann degradation of β-dihydromethine methiodide (V) gave a syrupy β-dihydronitrogen-free substance (VII) (Fig.

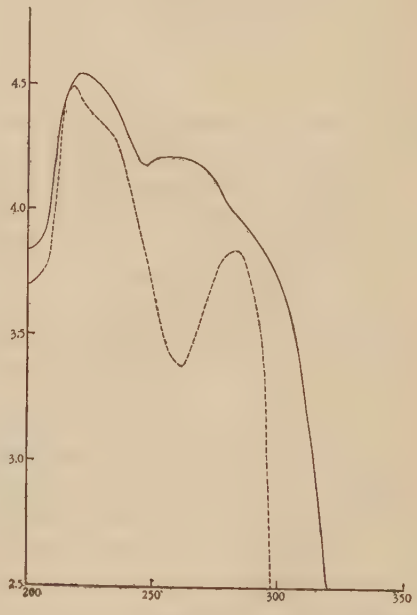
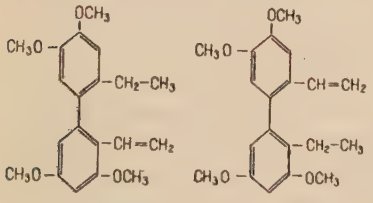


FIG. 2.
—— α-Dihydro-N-free substance (VI);
····· Tetrahydro-N-free substance (VIII).

1). Catalytic reduction of (VII) with palladiumcharcoal catalyst afforded the tetrahydro-substance of m. p. 58–60°, undepressed on admixture with (VIII) obtained from (VI).

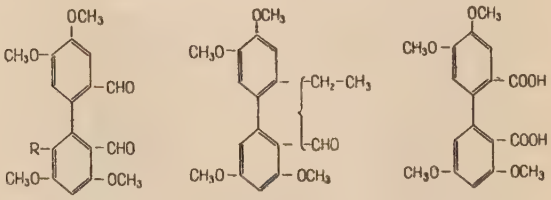
This proved that the difference of α- and β-form of the methylmethines lies in which side of -C-C-N-C-C- chain was splitted from N at the first stage of Hofmann degradation and moreover, that the remaining two side chains are quite similar, when the nitrogen atom was splitted off at the second stage of the Hofmann degradation.



(VI) and (VII) (VIII)

Ozonolysis of α-dihydro-N-free substance (VI) gave the monoaldehyde (XI), as pale yellow rhombic prisms of m. p. 107–108° with an yield of about 30%. It forms a monoxime of hexagonal plates, m, p. 169-171°.

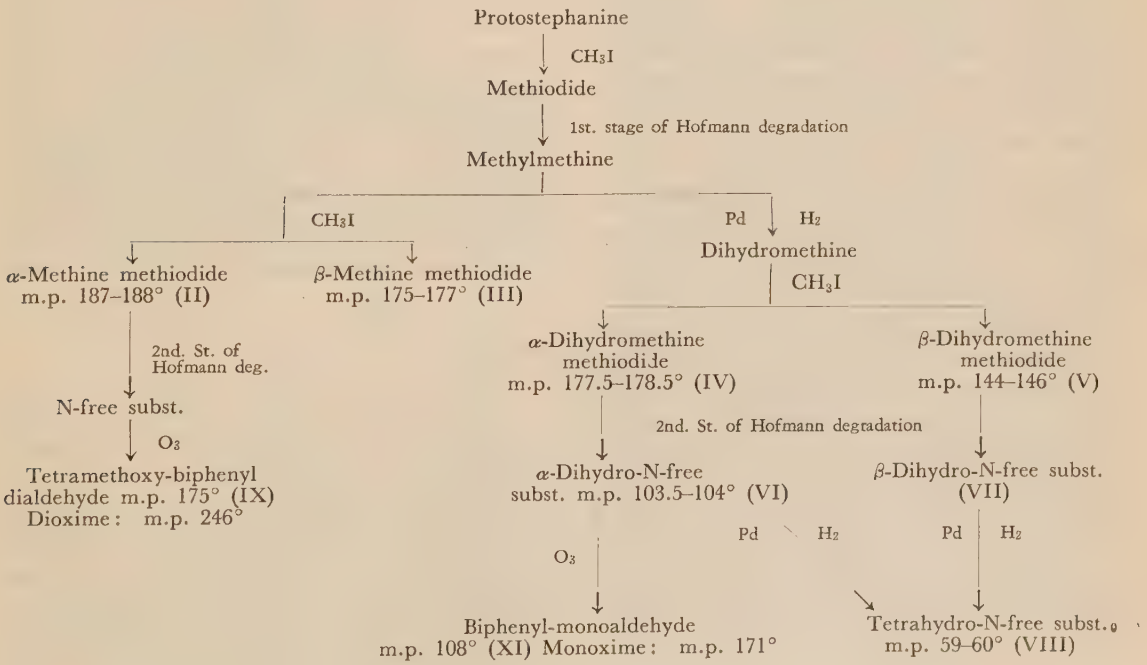
On the other hand, ozonolysis of N-free substance obtained by Hofmann degradation of bromoprotostephanine, which is relatively stable, afforded bromobiphenyl dialdehyde (X).[8]



(IX): R=H (XI) (XII)
(X): R=Br

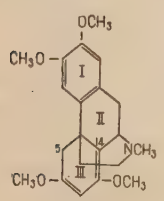
Previously the structure of protostephanine was considered as (XIII) or (XIV)[1] in spite of the fact that there was a high possibility of its structure to be a type of phenyldihydro-thebaine (XVIII), advocated by Robinson.[11]

8) H. Kondo and K. Takeda, *Ann. Rpt. ITSUU Lab,*, **5**, 51 (1954).

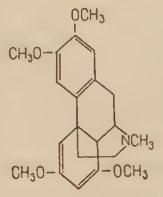


This idea seems to be more suspectable by the two kinds of methines (α- and β-isomer) were now isolated from the product of the first stage of Hofmann degradation.

The structure (XIII) or (XIV) for the base is to be rejected on the ground that the third ring of the base must be aromatic by the following reasons.



(XIII)



(XIV)

With the formula XIII or XIV the third ring of protostephanine, it must possess at least two double bonds from its molecular formula $C_{21}H_{27}NO_4$, and its ultraviolet absorption maximum at 284 mμ is log ε=3.80,[9] and the two methoxyl groups at C_6 and C_8 must be enolic.

The fact stand quite against those expectation, the suspected enol-ethers are not hydrolysed into ketones by warm dilute hydrochloric acid. The use of the strong acid effects only demethylation and the protostephanine is almost recovered from the demethylated product by further methylation.

These double bonds show strong resistance to catalytic hydrogenation with palladium-charcoal, platinum dioxide or by the skita hydrogenation.[6]

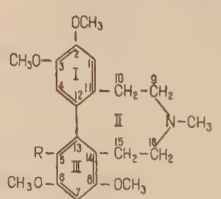
The action of bromine is a substitution at C_5 and not an addition at C_{14}.[1] Its formation of an adduct with maleic anhydride has recently entirely disproved.[1] Neither by Hofmann degradation nor by acetolysis, compounds belonging to the morphol series was not obtained, but the side chain always remained on the nucleus.

There are aporphine type and thebenine types in the phenanthren alkaloids, whose third ring is aromatic. But these two types were excluded in the case of protostephanine, on the ground that the base gives two kinds of methine by the Hofmann degradation, and that the base is optically inactive and could not be resolved into active forms.

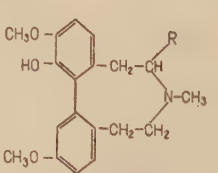
It becomes more natural, therefore, to assume a biphenyl type for protostephanine skeleton. By this assumption, the formation of biphenyl dialdehyde (IX and X by the ozonolysis of nitrogen-free substance of protostephanine and of bromoprotostephanine,[3,8] and also the formation of dicarboxylic acid by its oxidation with kalium permanganate, are adequately explained.

Substraction of tetramethoxybiphenyl, $C_{12}H_4(CH_3O)_4$, from the molecular formula of protostephanine, $C_{16}H_{12}(CH_3O)_4(NCH_3)$, would leave C_4H_8–NCH_3, and if approximately equal vinyl groups are to be formed by the first and the second stage of Hofamnn degradation, then the remainder must be in the form of -CH_2-CH_2-N(CH_3)-CH_2-CH_2-.

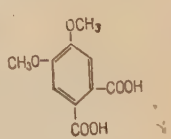
Its bonding in the first ring must be at C_{11}, because metahemipinic acid (XV) was obtained by the oxidation of protostephanine. The point of the attachment of this chain in the third ring can be no other than at C_{14}. This makes it inevitable to assume the type of the structure like phenyldihydrothebaine (XVIII) or α-methyldihydrothebaine (XIX), determined by Sir R. Robinson and K. W. Bentley,[10] and the structure of protostephanine is to be represented by (XVI). The



(XVI) R=H
(XVII) R=Br



(XVIII) R=C_6H_5
(XIX) R=CH_3



(XV)

ultraviolet spectra absorption curves of protostephanine and XVIII, XIX are similar as

9) G. Stork, *J. Am. Chem. Soc.*, **74**, 768 (1952).

10) K.W. Bentley and R. Robinson, *J. Chem. Soc.*, **1953**, 947.

shown in Fig. 3.

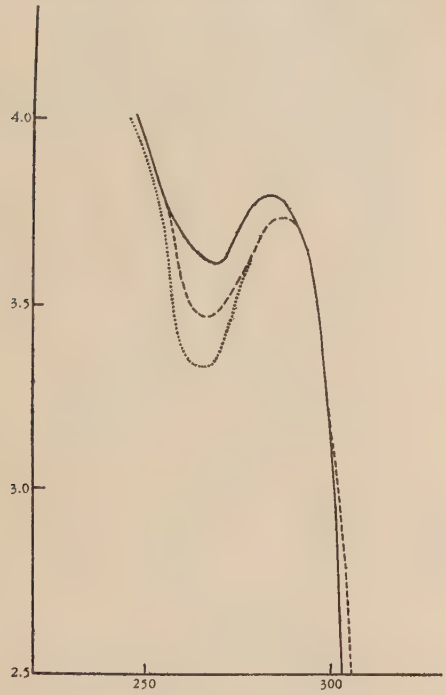
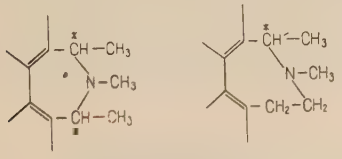


FIG. 3

——— Protostephanine (XVI).
– – – – Phenyldihydrothebaine (XVIII).
····· α-Methyldihydrothebaine (XIX).

Since protostephanine is optically inactive, configurations like the two shown below cannot be imagined.

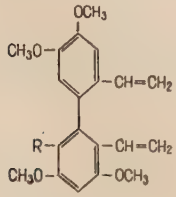
However, this biphenylstructure cannot lie on one plane so that the compound may



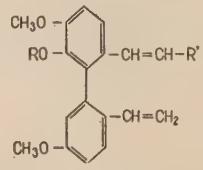
show some optical activity, but actually, the optical rotation is always zero, perhaps racemization in the case of no substituent on nine membered ring should be easy[10].

Application of bromine to XVI would naturally result in its substitution at C_5 and the bromoprotostephanine would then be represented by (XVII).

The nitrogen-free product of the exhaustive methylation of protostephanine would then be represented by the formula (XX), suggesting that the mutual effect of the two vinyl groups may cause facile polymerization. The nitrogen-free product (XXI) of phenyldihydrothebaine also undergoes polymerization to form a polymer of over 5000 in molecular weight.



(XX) R=H
(XXI) R=Br

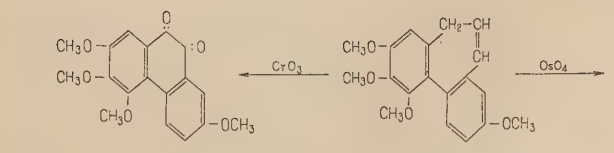


(XXII) R=H, R'=C_6H_5
(XXIII) R=CH_3, R'=CH_3

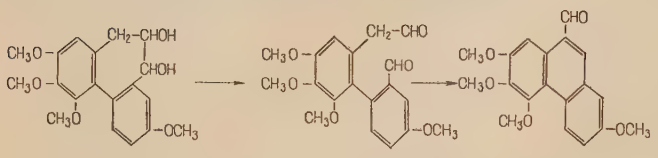
Ozonolysis of the nitrogen-free product (XXI) of bromoprotostephanine affords biphenyl dialdehyde (X)[8], and this formation would be explained conveniently by assuming formula (XVI) for it. The formation of biphenyl dicarboxylic acid by the permanganate oxidation of the N-free substance (XX) of protostephanine would be a same strain but this reaction was accompanied with the formation of phenanthraquinone (XXV),[3] which was also obtained by the oxidation of protostephanine methylammonium hydroxide (XXIV).[4] After, L. Small and E. Fry the oxidation of the methyl ether of N-free substance (XXIII) of α-methyldihydrothebaine with potassium bichromate had also afforded a quinone like compound as yellow crystals, m. p. 191–193°.[11]

Oxidation of deaminocolchinol methylether (XXVI), derived from colchicine, with chromium trioxide also yielded a quinone (XXVII), while stepwise oxidation of (XXVI) to the aldehyde (XXVIII) and condensation in the presence of a small amount of alkali carbonate has yielded phenanthraldehyde (XXIX) by Barton and others[12], and they supposed XXIX oxidized to XXVII. It may therefore be assumed that the permanganate oxidation of the methylammoniumhydroxide

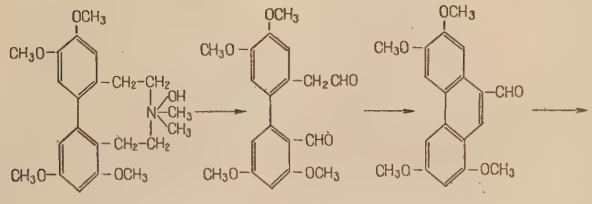
11) L. Small and E. Fry, *J. Org. Chem.*, **3**, 509 (1939).
12) N. Barton, et al, *J. Chem. Soc.*, **1945**, 176.



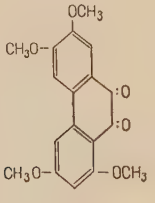
(XXVII) (XXVI)



(XXVIII) (XXIX)



(XXIV)



(XXV)

(XXVI) of protostephanine had also yielded the dialdehyde which underwent similar condensation to form the quinone (XXV).

A drastic oxidation of protostephanine itself[4] afforded metahemipinic acid alone. Since protostephanine is of biphenyl type, this oxidation could have given a phthalic acid from the third ring, but as this ring is a resorcinol ether, it might have been destroyed by the oxidation.

Formation of the tetramethoxyphenanthrene (I) by zinc dust-distillation must have occured during this process. Phenanthrene-derivative had also been obtained in the case of α-methyldihydrothebaine.[11] Compound of the morphol type had not been obtained, either by acetolysis or by the Hofmann degradation. This suggests that it would be more appropriate to assume that a phenanthrene skeleton is not present in the original base.

It is believed from the foregoing facts that the assumption of the formula XVI for protostephanine would be appropriate and able to explain all its properties and reactions without contradiction.

The alkaloids from *Stephania japonica* Miers, whose structures have been elucidated up to date, are stephanine[13] (aporphine type), epistephanine,[14] hypoepistephanine,[15] insularine[16] (biscoclaurine type), hasubanonine and homostephanoline (sinomenine type). It is noteworthy that another of its alkaloids, protostephanine, has now been shown to be a biphenyltype base, a modification of the morphine type, and that an alkaloid possessing two methoxyls, not in the adjacent position but, in meta-position had been found in nature. This biphenyl skeleton is similar to that of phenyldihydrothebaine which was obtained by Freund in 1905 by the action of phenylmagnesium bromide on thebaine, and whose structure has been studied by Smell and others, structurally determined by Robinson et al. in 1950.

EXPERIMENTAL

(A) Hofmann Degradation of Protostephanine

(1) Catalytic Reduction of Protostephanine Methylmethine. In a mixture of water and methanol,

13) T. Shirai, *J. Pharm. Soc. Japan*, **62**, 381 (1942); **64B**, 27 (1944).

14) H. Kondo and T. Tanaka, *ibid.*, **63**, 267, 273 (1943); **64**, 27 (1944).

15) H. Kondo and T. Nozoye, *ibid.*, **63**, 333 (1943).

16) M. Tomita and S. Uyeo, *J. Chem. Soc. Japan*, **64**, 147 (1943).

1.6 g of protostephanine methiodide was placed and silver oxide freshly prepared from 2.0 g of silver nitrate was added to turn the methiodide into the ammonium hydroxide. After evaporation of the solvent under a reduced pressure, the residue was dried at 110–115° under 4 mm Hg vacuum for 15 min. This residue was dissolved in ether, the ether solution was washed with water, and aqueous solution was evaporated. The residue was dried at 120–125° under 4 mm vacuum for 20 min. and again extracted with ether. The ethereal extracts were combined, washed with water, and extracted with dilute hydrochloric acid. The acid solution was rendered alkaline with ammonia and extracted with ether. The ether extract was washed with water, and the solvent evaporated, from which 1.2 g of syrupy methine was obtained. Elimination of treatment with dilute hydrochloric acid gave the same result.

A solution of 1.2 g of the foregoing methine dissolved in 20 ml of glacial acetic acid and added with 0.5 g of palladium-charcoal (20% Pd) was shaken in hydrogen stream 69.1 ml (calcd. 72.5 ml) of hydrogen were absorbed during 10 min. After shaking for 10 min. further, the catalyst was filtered off, and glacial acetic acid was distilled away under a reduced pressure. Water was added to the residue, basified with sodium carbonate, and extracted with ether. The ethereal extract was washed with water, dried, and evaporated, yielding 1.1g of syrupy dihydromethine.

(2) Dihydromethylmethine Methiodide. On the addition of 2.5 g of methyliodide to 1.0 g of the foregoing hydromethine, the mixture warmed spontaneously the whole turned into a crystalline mass. This was dissolved in sodium carbonate solution, washed with ether, and extracted with chloroform. The chloroform was evaporated and the residue was recrystallized from methanol to give α-dihydromethylmethine methiodide (IV) as rhombic crystals, m.p. 156–157° (effev.), containing methanol of crystallization. Recrystallization from hot water afforded hexagonal plates, m.p. 177.5–178.5°. Yield, 64%. *Analysis* Found: C, 53.53; H, 6.55; N, 2.67. Calcd. for $C_{23}H_{34}O_4NI$: C, 53.57; H, 6.65; N, 2.72%.

The mother liquor left after the removal of α-dihydromethyl-methine was concentrated and a small amount of ether was added in drops by which β-dihydromethylmethine methiodide (V) crystallized out. Recrystallization from methanol and ether afforded colorless rhomboprismatic crystals, m.p. 144–146°. Yield, 15%. *Analysis* Found: C, 53.48; H, 6.59: Calcd. for $C_{23}H_{34}O_4NI$: C, 53.57; H, 6.65%.

(3) α-Dihydro-N-free Substance (VI) from α-Dihydromethine Methiodide (IV). In a mixture of methanol and water, 1.0 g of (IV) was turned into the ammoniun hydroxide with silver oxide prepared from 1.5 g of silver nitrate. The filtered solution from silver was evaporated under a reduced pressure. The residue was dried at 115°, 4 mm Hg. for 15 min., during which an odor of trimethylamine was noticed. The residue was extracted with ether and the ethereal extract was washed with 1% hydrochloric acid and water. The dried ether was evaporated and the residue was recrystallized from methanol in colorless rhombic prisms, m.p. 103.5–104°. *Analysis* Found: C, 73.49; H, 7.42; CH_3O, 37.37. Calcd. for $C_{20}H_{24}O_4$: C, 73.13; H, 7.37; CH_3O, 37.81%.

(4) Tetrahydro-N-free Substance (VIII). A mixture of 0.16 g VI and 0.1 g of palladium-charcoal (Pd 20%) in 12 ml of glacial acetic acid was shaken in hydrogen stream and 11.4 ml (calcd. 10.8 ml) of hydrogen were absorbed during 13 min. After shaking for 30 min. further, the catalyst was filtered off, and glacial acetic acid was evaporated under a reduced pressure. Water was added to the residue, basified with sodium carbonate and was extracted with ether. After washing with water and drying, the ether was evaporated and the residue was recrystallized from methanol in 0.12 g of colorless rhombic prisms (VIII), m.p. 59–60°. *Analysis* Found: C, 72.78; H, 7.67. Calcd. for $C_{20}H_{26}O_4$: C, 72.68; H, 7.94%.

(5) β-Dihydro-N-free Substance (VII). To a suspension of the foregoing β-dihydromethine methiodide (VI) in aqueous methanol, silver oxide prepared from 0.15 g of silver nitrate was added and (VI) was converted in its ammoniumhydroxide. Silver was filtered off, the solvent was evaporated from the filtrate under the reduced pressure, and the residue was dried at 110°, 4 mm Hg., for 15 min., when an odor of trimethylamine was noticed. The residue was washed with 1% hydrochloric acid and water, and dried. Evaporation of ether left a syrupy β-dihydro-N-free substance, which showed somewhat tendency to polymerize.

This was catalytically reduced in glacial acetic acid with palladium-charcoal and rhombic prisms, m.p. 58–60° were obtained. This substance showed no depression of the melting point admixed with the tetrahydro-N-free substance (VIII) obtained from α-dihydro-N-free substance (VI).

(6) Ozonolysis of α-Dihydro-N-free Substance (VI). Oxygen containing 6% of ozone was passed through the solution of 0.46 g of (VII) in dehydrated chloroform until saturation. Excess of ozone was removed by bubbling dry air through this solution and chloroform was evaporated at room temperature under a reduced pressure. Water was added to the pale yellow, syrupy residue and gradually warmed to 60° to decompose the ozonide.

Ethanolic solution of dimedone was added to the aqueous layer and 0.13 g of formaldimedone, m.p. 190–191°, was obtained, corresponding to 0.32 molar equivalent of formaldehyde.

The syrupy layer was taken up in ether and the ether extract was washed with water and dried. The residue obtained on evaporation of ether was crystallized from a mixture of acetone and petroleum ether (up to 40°), but the majority remained as a reddish syrup. The crystals were recrystallized from acetone and 0.11 g (23%) of biphenyl monoaldehyde was obtained as pale yellow plates, m.p. 107–108°. *Analysis* Found: C, 68.43, 68.79; H, 6.47, 6.12. Calcd. for $C_{19}H_{22}O_5$: C, 69.04; H, 6.72%.

Oxime: A mixture of the foregoing aldehyde and methanol solution of hydroxylamine was warmed for 10 min. by which crystals separated out and were recrystallized from methanol to colorless octagonal plates, m.p. 169–171°. *Analysis* Found: C, 65.62; H, 6.18; N, 3.96. Calcd. for $C_{19}H_{22}O_4$ (NOH): C, 66.05; H, 6.72; N, 4.06%.

(7) β-Methylmethine. The syrupy methylmethine obtained in (1) was added with methanolic solution of picric acid. The picrate was obtained as a rhombic prisms, m.p. 166–167°. Liberation of the methine with alkali and conversion to the methiodide yielded α-methylmethine methiodide (V), m.p. 187–188°.

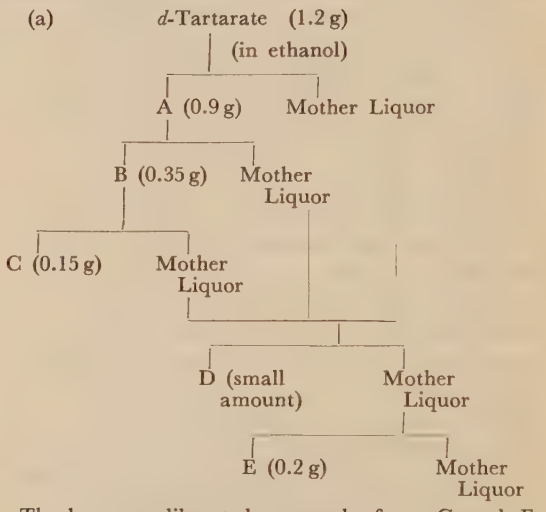
The mother liquor left after the removal of the foregoing picrate did not crystallize. But the methine liberated from it with alkali, gave a methiodide in a small amount, which recrystallized from methanol in rhombic prisms, melted at 175–177°. *Analysis* Found: C, 54.11; H, 6.00. Calcd. for $C_{23}H_{32}O_4NI$: C, 53.73; H, 6.29%.

(B) Attempted Resolution of Protostephanine with *d*-Tartaric Acid and *d*-Benzoyl-tartaric Acid.

(1) Resolution of *d*-Tartarate. *d*-Tartarate of protostephanine was prepared and dissolved in ethanol with warming and the solution somewhat evaporated to allow each salt to crystallize out. The crystals that precipitated out were somewhat long, rhombic prisms of m.p. 178–179° and the presence of antipode could not be observed. The base liberated from the crystals with ammonia was submitted to optical rotation measurement but gave $(\alpha)_D = 0°$, at which the error was around ±1–2°.

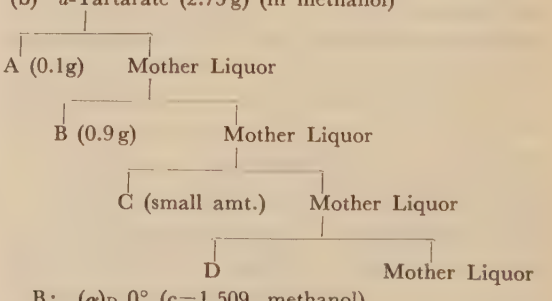
Ethanol was found to be the best solvent for crystallization and acetone, water and methanol were not suitable. Crystallizing out under ice-chilling also failed to effect resolution.

A few examples of the crystallization will be shown below:



The base was liberated separately from C and E with ammonia and optical rotation of each was measured.

C: (α) 0° (±1.6°) (c=1.20, ethanol).
E: (α) 0° (±2.1°) (c=0.81, ethanol).



B: $(\alpha)_D$ 0° (c=1.509, methanol).
D: $(\alpha)_D$ 0° (c=0.82, methanol).

(2) Resolution as Dibenzoyl-*d*-tartarate. The dibenzoyl-*d*-tartarate easily crystallized out as rhombic prisms with pointed ends, melting at 144°. Several

recrystallization as in the foregoing experiments failed to yield any optically active compounds.

The Author takes this oppotunity to extend his deep gratitude to Dr. H. Kondo, Professor Emeritus of the University of Tokyo and the director of this Laboratory, who has given him kind guidance and encouragement throughout the course of this work. Author's thanks are due to Mr. K. Watanabe of this laboratory for the ultraviolet spectral measurement and elementary analysis and to the Ministry of Education for the Grant in Aid of Developement of Scientific Research, which made him possible to carry out this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 174~177, 1956]

Studies on Amino Acids. II

Studies on the Enzymatic Resolution. (II) Enzymatic Resolution of DL-Lysine (1)*

By Ichiro CHIBATA, Shigeki YAMADA and Shun'ichi YAMADA

*Osaka Research Laboratory, Gohei Tanabe & Co., Ltd.***

Received May 25, 1956

The enzymatic procedures for the resolution of DL-lysine such as asymmetric synthesis of acyl L-lysine anilide and asymmetric hydrolysis of acyl DL-lysines have been studied. As a result, the procedure consisting in the enzymatic asymmetric hydrolysis of ε-benzoyl-α-acetyl-DL-lysine was found to be the most advantageous for the resolution of DL-lysine.

In the previous paper[1], the authors reported a simple method of enzymatic resolution of DL-methionine. Recently, lysine, one of the indispensable amino acids, has been found to play an important role in pharmaceutical and nutritional fields. The authors have carried out the study of enzymatic resolution of DL-lysine in order to establish a superior method of the preparation of L-lysine, and obtained a satisfactory result.

The first resolution of DL-lysine into its optical antipodes was carried out through the chemical procedure by Berg[2], in 1936. Since, along with the development of enzymatic methods of resolution in recent years, a number of reports on the resolution of DL-lysine employing enzymatic procedures such as the asymmetric synthesis of acyl L-lysine anilides[3-10] and asymmetric hydrolysis of acyl DL-lysine derivatives[10-16] have been presented. Neither of these methods was, however, found completely satisfactory in respect of the enzyme sources or preparation of the substrates.

The results hitherto reported on the enzymatic synthesis of acyl L-lysine anilides are summarized in Table I. The authors, also, investigated the asymmetric anilide synthesis with papain from acyl DL-lysines such as α, ε-diacetyl-, ε-acetyl-α-benzoyl-, ε-acetyl-α-chloroacetyl-, ε-benzoyl-α-acetyl α, ε-dibenzoyl-, ε-benzoyl-α-chloroacetyl, and α, ε-dichloroacetyl-derivatives. The general procedure employed for the synthesis of acyl L-lysine anilides was as follows. A mixture of acyl derivative (M/100), N-NaOH (M/100) and distilled water (15 ml) was warmed on a water-bath to dissolve. Then aniline (M/100) and Mcilvaine's citrate buffer (M/5, pH 5.0, 6 ml) were added, and the pH of the mixture was adjusted to 5.0 if necessary. The enzyme solution (15 ml), prepared by extracting papain with a tenfold volume of water containing a small amount

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** Honjo-kawasaki-cho, Oyodo-ku, Osaka.

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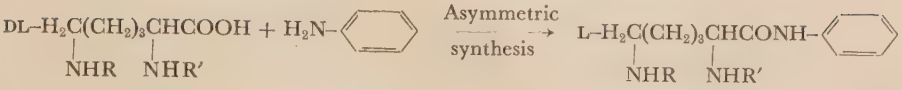
TABLE I

ASYMMETRIC SYNTHESIS OF ACYL L-LYSINE ANILIDES



Acyl Derivs.*		Acyl L-Lysine Anilides (Yield, %)			
(R)	(R′)	Papain[7]	Papain[8]	Ficin[9]	Papain**
Ac.	Ac.		0		0
Ac.	Clac.				0
Ac.	Bz.				0
Clac.	Clac.				0
Bz.	Form.			0	
Bz.	Ac.	53		0	43.5
Bz.	Clac.			0	0
Bz.	Bz.	100	50(DL)	99	89.7
Cbz.	Cbz.	100[9]	5		
Prop.	Prop.		60		
Butyr.	Butyr.		95		
i-Butyr.	i-Butyr.		100		
Capr.	Capr.		100		
i-Capr.	i-Capr.		100		

* Ac.: acetyl, Clac.: chloroacetyl, Bz.: benzoyl, Form.: formyl, Cbz.: carbobenzoxy, Prop.: propionyl, Butyr.: butyroyl, Capr.: caproyl.

** The results obtained by the authors.

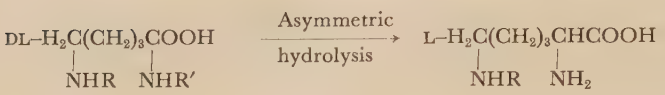
of potassium cyanide so as to activate papain, was introduced, and the mixture incubated at 37°C for several days. Of the acyl DL-lysines investigated, α, ε-dibenzoyl-, and ε-benzoyl-α-acetyl-derivatives separated the anilides in 89.7% and 43.5% yields (calculated from L-antipode), respectively. Other acyl-derivatives failled to give crystalline anilide. α, ε-Dibenzoyl-L-lysine anilide thus obtained was, however, contaminated with D-antipode and shows low optical purity. On the other hand, although ε-benzoyl-α-acetyl-L-lysine anilide showed high optical purity, the yield was not satisfactory. Judging from the results obtained by the authors, it appears that the method of resolution by asymmetric anilide-synthesis is not appropriate for the preparation of L-lysine.

As for the other enzymatic procedure, asymmetric hydrolysis of acyl DL-lysines, the hitherto reported results are listed in Table II. As shown in the Table, the rate of hydrolysis of acyl DL-lysines by hog kidney enzyme is much lower than that of acetyl-DL-methionine, and Greenstein and his co-workers succeeded in the resolution of acyl DL-lysines only when a large amount of enzyme preparation or a highly purified enzyme was employed. Although Neuberg's result appears satisfactory, the preparation of the substrate, α-benzoyl-DL-lysine, is inconvenient.

The authors investigated the asymmetric hydrolysis of various acyl DL-lysines by the enzyme prepared from *Aspergillus Oryzae* in order to find out the most advantageous substrate. The procedure essentially follows the previous paper. The acyl DL-lysine (M/100) was dissolved in N-NaOH (M/100) and Sörensen's phosphate buffer (M/15, pH 7.5, 10 ml). To this solution, enzyme solution prepared by extracting the Taka-diastase (1 g)

TABLE II

ASYMMETRIC HYDROLYSIS OF ACYL DL-LYSINES



Acyl Derivs. (R)	(R')	12,13) Hog kidney Homogenate	13,14) Acylase I	11) Hog Kidney Homogenate	15) Taka-diastase
Ac.	Ac.		70 μM*, 68%		
—	Clac.	6–10 μM			
Clac.	Clac.	10 μM, 78%	140 μM		
Bz.	Form.			0	
Bz.	Ac.			0	
Bz.	Clac.	6–10 μM		72%	
—	Bz.				80%
Bz.				0	
Acetyl-DL-methionine		615 μM	24200 μM		

* μM of hydrolyzed substrate/hour/mg of Prot. N

with ten fold water was added. The pH of the digestion mixture was adjusted to 7.5 if necessary, filled up to 100 ml with distilled water, and incubated at 37°C. To observe the susceptibility of acyl DL-lysines, the extent of hydrolysis was determined by measurement of the liberated amino acid by the Van Slyke procedure. The data obtained indicates that the rate of hydrolysis of acyl-derivatives by the enzyme decreases for the various substituent groups in the following order ε-benzoyl-α-acetyl > α,ε-dichloroacetyl = ε-benzoyl-α-chloroacetyl ≥ α, ε-dibenzoyl > ε-acetyl-α-benzoyl > ε-acetyl-α-chloroacetyl > α, ε-diacetyl. It is of interest to note that not only the nature of the substituent groups in α-position but the combined effect with the acyl group in ε-position, has also an important influence upon the rate of hydrolysis. As in the case of Acylase I reported by Greenstein[13], dichloroacetyl derivative is hydrolyzed at a faster rate than the corresponding diacetyl derivative, namely, the former was hydrolyzed to 71.8% add the latter to 13.6% after 96 hours' incubation under the conditions employed by the authors.

In this study on the hydrolysis of a series of acyl DL-lysines, ε-benzoyl-α-acetyl-DL-lysine was found to be the most advantageous substrate. According to the report by Utzino[11], this compound was not hydrolyzed by the hog kidney homogenates, although, this derivative is most rapidly hydrolyzed with the enzyme prepared from *Aspergillus* in the author's experiments. Namely, the L-antipode of the substrate was hydrolyzed almost quantitatively within 80 hours by using such a small amount of the enzyme as onetwentieth weight of Taka-diastase to the substrate. Furthermore the hydrolyzed product, ε-benzoyl-L-lysine, separated as crystals from the digestion solution during the incubation process. Therefore, ε-benzoyl-L-lysine of high purity could be easily obtained by filtration in 90% yield. ε-Benzoyl-L-lysine thus obtained was chemically hydrolyzed by the method employed for the preparation of the racemic compound to L-lysine dihydrochloride, which readily converted to the monohydrochloride by the treatment with a weak basic ion-exchange resin, Amberlite IR-4B, resulting in a good yield. While, the unhydrolyzed

antipode of the substrate, ε-benzoyl-α-acetyl-D-lysine, was recovered and converted to the racemic form by the treatment with acetic anhydride. The substrate thus recovered could be used for the successive run.

In this method, the substrate is readily available, that is, ε-benzoyl-α-acetyl-DL-lysine is easily obtained as crystals from the intermediate in the synthesis of DL-lysine, ε-benzoyl-DL-lysine. A further advantage of using this substrate is that the ordinary employed organic solvent extraction for the separation of respective antipodes after enzymatic hydrolysis is unnecessary for the L-antipode is nearly separated by filtration. So, it may be said that this procedure, the asymmetric hydrolysis of ε-benzoyl-α-acetyl-DL-lysine by the *Aspergillus* enzyme preparation provides the most convenient and superior method for preparing the optically active lysine.

EXPERIMENTAL

ε-Benzoyl-α-acetyl-DL-lysine: To a solution of ε-benzoyl-DL-lysine (12.5 g) dissolved in 2N-NaOH (25 ml) and water (40 ml), acetic anhydride (12 ml) were added alternatively under stirring at 5°, then additionally stirred for one hour at room temperature. The reaction mixture was acidified with 6N-HCl (70 ml) and allowed to stand overnight in a refrigerator. The separated crude ε-benzoyl-α-acetyl-DL-lysine was collected and after recrystallization from water gave colorless needles, m.p. 144–145°; yield, 10.5 g (72.0%)

Enzymatic Hydrolysis of ε-Benzoyl-α-acetyl-DL-lysine: ε-Benzoyl-α-acetyl-DL-lysine (14.6 g) was dissolved in N-NaOH (50 ml) and 2M/15 phosphate buffer, pH 7.5 (100 ml). The enzyme solution (7.5 ml) prepared by extracting Taka-diastase with tenford weight water, was added to the solution. The mixture was adjusted to pH 7.5 if necessary and filled up to 230 ml with distilled water. The digestion mixture was incubated at 37° for 80 hours. Crystals of ε-benzoyl-L-lysine separated during the incubation were collected by filtration then washed with alcohol; yield, 5.6 g. (89.5%) m. p. 266–268°, $[\alpha]_D^{25}=+17.2°$ (3% in 6N-HCl).

L-Lysine Monohydrochloride: ε-Benzoyl-L-lysine (5.6 g) was refluxed with 6N-HCl (56 ml) for 8 hours. After being chilled, the separated benzoic acid was filtered off. The filtrate was evaporated in vacuo, and the residue was dissolved in water and evaporated again to remove excess hydrochloric acid. The residue was dissolved in water (100 ml) and decolorized. This solution was passed through the column packed with Amberlite IR-4B. The effluent which gave positive ninhydrin reaction test was collected and adjusted to pH 4.2 with hydrochloric acid. After being concentrated, absolute alcohol was added and it was allowed to stand overnight in a refrigerator. The separated L-lysine monohydrochloride was collected by filtration, washed with alcohol then dried; yield, 3.42 g. (83.5%), m. p. 256–260° (dec.), $[\alpha]_D^{25}=+20.8°$ (3% in 5N-HCl). *Anal.* Found: C, 39.30; H, 8.51; N, 15.19, Calcd. for $C_6H_{15}N_2O_2Cl$: C, 39.46; H, 8.28; N, 15.34.

Although L-lysine monohydrochloride is also obtained by the addition of pyridine to the alcoholic solution of the residue obtained by concentrating the filtrate of the reaction mixture, the former procedure employing anion exchange resin was found to be advantageous.

ε-Benzoyl-α-acetyl-D-lysine and its Racemization: After the removal of ε-benzoyl-L-lysine from the digestion mixture, the filtrate was concentrated in vacuo, acidified with hydrochloric acid, and following extraction with ethyl acetate gave ε-benzoyl-α-acetyl-D-lysine. However, the racemization procedure is more convenient for the preparative purpose of L-lysine. That is, the mother liquor and washings from the preparation of ε-benzoyl-L-lysine were combined and evaporated at reduced pressure. The residue was dissolved in 2N-NaOH (12.5 ml) and water (15 ml), and acetic anhydride (25 ml) added. The mixture was stirred at 40–50° for 3 hours, then kept at 40° for 20 hours for the purpose of racemization. The reaction mixture was concentrated and the residue was taken up in water, acidified with hydrochloric acid, and allowed to stand overnight in a refrigerator yielding crude ε-benzoyl-α-acetyl-DL-lysine (4.2 g), which showed no optical rotation indicating complete racemization. After being recrystallized from water the recovered substrate showed no melting point depression when mixed with the authentic sample, and could be used for the next run.

The authors are grateful to Prof. H. Mitsuda of Kyoto University and Dr. M. Fujisawa, Director of this Laboratory, for their encouragement during the course of this study.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 178~182, 1956]

The Determination of D-Glutamic Acid and D-Aspartic Acid by Means of a New Oxidase

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Received June 6, 1956

The fractional determination of D-glutamic and D-aspartic acids using the enzyme preparation of *Aspergillus ustus* strain f. was studied. In the first part of this paper, the procedure of enzyme preparation, the effect of sodium chloride on enzyme activity, and a new device for the fractional determination of D-glutamic and D-aspartic acids are described. In the latter part, the contents of D-glutamic and D-aspartic acids of cancer and normal tissues are estimated. However, it was found that the cancer tissues are not characterized by the presence of D-glutamic acid in opposition to Kögl's claim.

In the previous report[1], the method for the manometric determination of D-glutamic acid was presented. Since it has been made clear that the enzyme preparation oxidizes D-aspartic acid as well as D-glutamic acid, specific determination of the latter should be difficult in the presence of the former. In the present study fractional determination of D-glutamic and D-aspartic acids was investigated. Determination of D-glutamic and D-aspartic acids contents of cancer and normal tissues by application of the manometric method is also reported in this paper.

The Principle of the Determination

The dried cells or the cell-free enzyme preparation of *Aspergillus ustus* strain f. oxidizes D-glutamic and D-aspartic acids according to the following equations:

$$\text{D-glutamic acid} + \frac{1}{2}O_2 \longrightarrow \alpha\text{-ketoglutaric acid} + NH_3$$

$$\text{D-aspartic acid} + \frac{1}{2}O_2 \longrightarrow \text{oxalacetic acid} + NH_3$$

These reactions, however, proceed only when the enzyme preparation possesses catalase activity[2]. In case a sample contains one of these D-amino acids, the amount of this D-amino acid can be determined manometrically by measuring the oxygen uptake. If a sample contained both D-glutamic and D-aspartic acids, it is also possible to determine these D-amino acids separately by estimating the amounts of α-ketoglutaric acid and oxalacetic acid in the reaction mixture, after oxygen consumption is completed.

Enzyme Preparation

All of the dried cells, the crude extracts and the partially purified enzyme preparation are able to be used for the determination. The dried cells or the crude extracts, however, have not only a relatively high rate of endogenous respiration which is often affected by materials contained in the sample, but also a weaker activity towards D-alanine. Therefore, it will be better to use the partially purified enzyme preparation for determination. An example of enzyme preparation is described below. The mycelia of *Aspergillus ustus* strain f. incubatd in a medium containing crude glucose 2%, D-glutamic acid 0.5%, K_2HPO_4 0.2%, and $MgSO_4 \cdot 7H_2O$ 0.1% in a one to one mixture of distilled water and tap water for 3–5 days on a shaker or in an aerobic jar fermenter were employed. The mycelia were washed with distilled water several times and stored at −20°C for 3~24 hours and dried over $CaCl_2$ in vacuum. Four grams

* Institute of Applied Microbiology, University of Tokyo.

1) S. Mizushima, K. Izaki, H. Takahashi, and K. Sakaguchi This Bulletin, **20**, No. 1, 36 (1956).

2) S. Mizushima, and K. Sakaguchi, This Bulletin, **20**, No. 3, (1956).

of the dried mycelia was ground in a mortar and then extracted with the borate-phosphate buffer (pH 8.0). Eighty ml of the crude extracts obtained were frozen at −20°C overnight and centrifuged in order to remove the precipitate. To the supernatant solution solid ammonium sulfate was added until a concentration of 60% saturation was reached. The precipitate was recoverd by centrifugation, resuspended in 20 ml of the borate-phosphate buffer (pH 8.0).

This solution was used as the enzyme solution. This preparation is so stable that storage at −20°C for a few months is possible. This enzyme preparation strongly oxidizes D-glutamic and D-aspartic acids, but does not affect any of the other DL-amino acids,

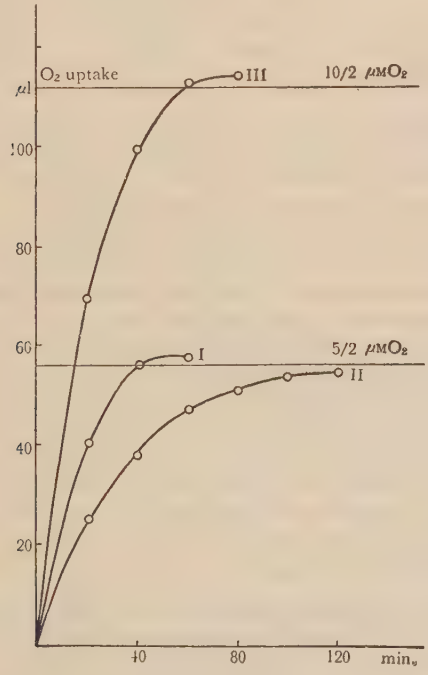

FIG. 1. Oxidation of D-glutamic and D-Aspartic Acids by the Enzyme Preparation.

Each vessel contained 0.4ml of the enzyme preparation and borate-phosphate buffer (pH 8.0) in the main compartment, the substrate (Curve I 5μM of D-glutamate, Curve II 10μM of DL-aspartate, and Curve III 5μM of D-glutamate plus 10μM of DL-aspartate) in the side arm, and 0.2ml of 20% KOH in the centre well; total volume 2.4 ml.

α-ketoglutaric acid and oxalacetic acid at all. By using this enzyme preparation, D-glutamic and D-aspartic acids were oxidized quantitatively as shown in Fig. 1.

Effect of Sodium Chloride and other Inorganic Salts on the Determination. The effect of sodium chloride on the enzyme activity and on the final volume of oxygen uptake for 5μM of D-glutamic and 10μM of DL-aspartic acids, are shown in Figs. 2, 3 and 4 respectively. When the concentration of sodium

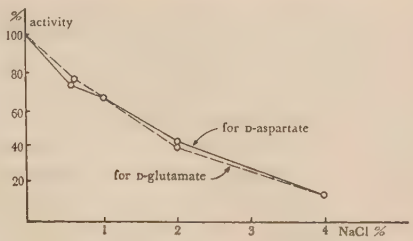

FIG. 2. Effect of Sodium Chloride on the Enzyme Activity.

Effect of sodium chloride on enzyme activity was observed by measuring the rate of oxygen consumption with the Warburg manometer. Each vessel contained the enzyme preparation, sodium chloride, and the borate-phosphate buffer (pH 8.0) in the main compartment, 0.2 ml of M/10 D-glutamate or M/5 DL-aspartate in the side arm, and 0.2ml of 20% KOH in the centre well; total volume 2.2ml. Readings were taken for 20 minutes.

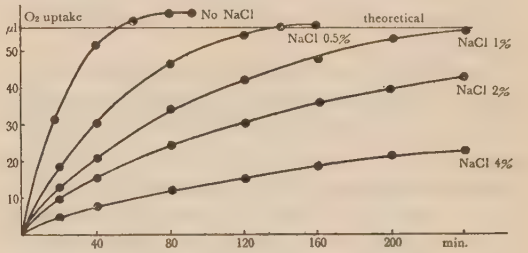

FIG. 3. Effect of Sodium Chloride on the Determination of D-Glutamic Acid.

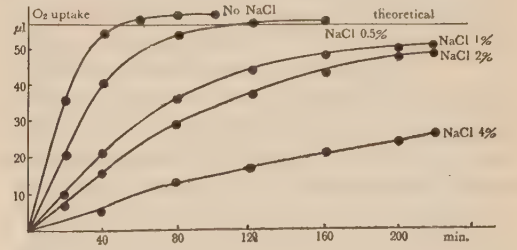

FIG. 4. Effect of Sodium Chloride on the Determination of D-Aspartic Acid.

In Fig. 3 and Fig. 4, each vessel contained 0.4 ml of the enzyme preparation, sodium chloride, and the borate-phosphate buffer (pH 8.0) in the main compartment, 0.2 ml of M/40 D-glutamate or M/20 DL-aspartate (5μM as D-amino acid in both cases) in the side arm, and 0.2ml of 20% KOH in the centre well; total volume 2.2 ml.

chloride in the reaction mixture was higher than one percent, a long time was required to complete the enzymatic reaction, and often, the volume of the oxygen uptake did not reach the theoretical. In order to study whether this inhibitory action was caused by the chloride ion, by the sodium ion, or by the action of a strong electrolytic salt, a similar experiment was carried out for potassium chloride, sodium sulphate, and potassium sulphate. It was found that the oxidase was inhibited by potassium chloride to the same extent as by sodium chloride, whereas sodium sulphate and potassium sulphate showed weaker but considerable inhibitory actions towards the enzyme activity at the same concentration of moles. Therefore the inhibitory effect of sodium chloride on enzyme activity seems to be caused, partially by the chloride ion and partially by the action of strong electrolytic salt. In consideration of these facts, it is desirable to maintain the concentration of sodium chloride in a reaction mixture less than 0.5%.

Fractional Determination of D-Glutamic and D-Aspartic Acids. When the sample contains D-glutamic and D-aspartic acids, it is necessary to determine α-ketoglutaric acid and oxalacetic acid, the oxidative products of D-glutamic and D-aspartic acids in the reaction mixture, since both D-amino acids are oxidized by the same enzyme preparation. Oxalacetic acid, however, is easily decarboxylated to give pyruvic acid spontaneously. Therefore, the fractional determination of α-ketoglutaric acid and pyruvic acid should be carried out after converting the residual oxalacetic acid to pyruvic acid with aluminum sulfate. The fractional determination of α-ketoglutaric acid and pyruvic acid has been reported by many investigators. In this paper, these organic acids were determined colorimetrically as 2,4-dinitrophenylhydrazone. An example of the fractional determination of D-glutamic and D-aspartic acids is shown below. First, the total amount of D-glutamic and D-aspartic acids are determined with the Warburg manometer at 30°C by measuring the oxygen uptake. The vessel contains the enzyme preparation and M/15 borate-phosphate buffer (pH 8.0) in the main compartment, 0.2–0.4ml of a sample solution in the side arm and 0.2 ml of 20% potassium hydroxide solution in the centre well with 1cm² of filterpaper. The experiment is carried out in paralell with a control experiment and readings are taken for 1–2 hours until the pressure changes in the control and experimental flasks become equal to each other. Then, to the reaction mixture, are added 0.75 M phosphate buffer and 33.3% aluminum sulfate solution to convert oxalacetic acid to pyruvic acid according to the method of Krebs and Eggleston[3].

After standing for one hour at 30°C, the fractional determination of α-ketoglutaric acid and pyruvic acid is carried out by the method of Friedemann and Haugen[4].

The values given in Table I show the results for the mixture of 5μM of D-glutamic acid and 10μM of DL-aspartic acid.

TABLE I

FRACTIONAL DETERMINATION OF D-GLUTAMIC ACID AND D-ASPARTIC ACID

	D-glut.+ D-asp.	D-glutamic acid	D-aspartic acid
Theoretical	10.0μM	5.0μM	5.0μM
Experimental	10.2	4.2	5.0

Discussion. In this paper, the direct microdetermination of D-glutamic and D-aspartic acids has been demonstrated for the first time. Though the total amount of D-glutamic and D-aspartic acids is determined accurately, the fractional determination of D-glutamic and D-aspartic acids produces a relatively high degree of error. The errors were chiefly caused by unaccuracy of the fractional determination of α-ketoglutaric acid and pyruvic acid. Figs. 2, 3, and 4, show that the concentration of sodium chloride in the reaction mixture has a great influence upon enzymatic determination. When acid hydrolysate of protein is used as a sample, it is necessary to expel the excess hydrochloric acid from the hydrolysate as far as possible by concentrating to dryness several times under reduced pressure before neutralization with sodium hydroxide.

THE DETERMINATION OF D-GLUTAMIC AND D-ASPARTIC ACIDS CONTENTS OF CANCER AND NORMAL TISSUES

Previous reports by Kögl and co-workers[5] on the characteristic occurrence of D-glutamic acid in malignant tumors in man and rabbit have been discussed by several authors[6], but

3) H.A. Krebs and L.V. Eggleston *Biochem. J.* **39**, 408. (1945).

4) T.E. Friedemann and G.E. Haugen. *J. Biol. Chem.* **149**, 415 (1943).

5) F. Kögl, and H. Erxleben, *Z. Physiol. Chem.* **258**, 57 (1939), **261**, 54 (1939), **263**, 107 (1940).

6) A.C. Chibnall, et al. *Nature*, **144**, 71 (1939), E. Chargraff. *J. Biol. Chem.* **130**, 29 (1939), S. Graff, *J. Biol. Chem.* **130**, 13 (1939), S. Graff, D. Rittenberg, and G.L. Foster, *J. Biol. Chem.*, **133**, 745 (1940), J. Wieland and W. Paul, *Ber.*, **77**, 34 (1944) etc.

TABLE II

DETERMINATION OF D-GLUTAMIC AND D-ASPARTIC ACIDS CONTENTS OF CANCER AND NORMAL TISSUES

Column 1	2	3	4	5	6
Material	Crude-protein	L-glutamic acid*	D-glutamic acid +D-aspartic acid	Column 4 / Column 3+4 × 100	Recovery of D-glutamic acid
Normal tissues:	mg	mg	mg	%	%
Muscle (horse)	1000	59.5, 57.9	1.3, 1.4	2.2±0.1	104
〃 (rat)	670	77.1, 73.8	1.7, 1.4	2.0±0.2	71
Liver (mouse)	230	27.0, 26.9	0.4	1.5	92
Tumor tissues:					
N-F sarcoma (mouse)	270	27.5, 27.1	0.6, 0.8	2.5±0.5	99
Rhodamine sarcoma (rat)	400	42.3, 44.3	1.4	3.1	99
Same, necrotic part	100	13.3	0.4	2.9	97
Ehrlich ascites carcinoma (mouse)	580	60.1, 57.4	0.5, 0.4	0.7+0	108
Ehrlich solid carcinoma	430	44.7, 46.8	1.8, 1.7	3.7±0.2	107
Ehrlich ascites fluid	840	101.2, 90.2	1.6, 2.2	1.9±0.4	90
Ascitic hepatoma (rat)	300	36.6, 33.6	2.6, 2.7	6.9±0.5	95
Primary liver carcinoma (man)	1600	96.0, 96.0	2.6	2.7	105
Kidney adenocarcinoma (man)	1000	70.6, 64.2	2.0	2.9	78

* L-Glutamic acid was determined manometrically, using L-glutamic acid decarboxylase from *Escherichia coli crooks*.

the majority of them failed to confirm the results obtained by Kögl. However, strictly speaking, isolation procedures or the isotopic dilution method employed by these authors are not adequate to prove that their results are really contradictory to the conclusion of Kögl. So far as we know, the direct determination of D-glutamic acid content of tumors has hitherto never been reported. We have, therefore, determined D-glutamic and D-aspartic acids contents of the hydrolysate of cancer and normal tissues by the manometric method.

Preparation of Amino Acid Solution from Normal and Cancer Tissues. Amino acid solutions were prepared according to the procedure described by Kögl. Tissue was prepared in the Waring Blender with 0.6% sodium chloride solution of the volume six times as large as the tissue, and stored at 0°C for 24 hours. After centrifugation the salt solution was mixed with four volumes of ethanol. The resulting precipitate was washed with 80% ethanol and was heated at 110°C to dryness. It was then hydrolysed for 7 hours in a 20% hydrochloric acid solution of the weight twenty times as large as the tissue. From the hydrolysate, excess hydrochloric acid was expelled as far as possible by concentrating to dryness several times under reduced pressure.

The sample was neutralized in aqueous solution and diluted, and was used in the experiment.

Results and Discussion

Determination of L-glutamic acid and the total amount of D-glutamic and D-aspartic acids were carried out for three kinds of normal tissue and nine kinds of cancer tissue. The results are shown in Table II. The values in column 5 show the ratio in percentage of the total amount of D-glutamic and D-aspartic acids to that of DL-glutamic and D-aspartic acids. As the total amount of D-glutamic and D-aspartic acids was very small, the fractional determination of these D-amino acids was not carried out. Therefore, the ratio in percentage of D-glutamic acid to total glutamic acid must be smaller than these values. Except for ascitic hepatoma of the rat, all values of column 5 are below the level of 4%. Since it has been widely accepted that 3–6% of L-glutamic acid is racemized by treatment with hydrochloric acid at 100°C for several hours, the presence of D-glutamic acid by 4% does

not indicate the occurrence of D-glutamic acid in the original tissue. As to the ascitic hepatoma from rat, though the value in column 5 does not seem to be very small, this value is still not so large as that reported by Kögl. The small and uniform values of D-glutamic acid observed in the variety of hydrolysate examined for normal and cancer tissues, show that cancer tissues are not characterized by the presence of D-glutamic acid.

We are deeply indebted to Drs. W. Nakahara, K. Oota, F. Fukuoka, and T. Sugimura of the Cancer Institute, the Japanese Fundation of Cancer Research, for supplying us with cancer tissues used and for thir advice. Our thanks are also due to Assist. Prof. H. Yonehara and Mr. H. Yamazaki of the Institute of Applied Microbiology, University of Tokyo, for Ehrlich carcinoma of mouse.

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Studies on the Conjugated Lipids*

Part V. Configuration of the Galactoside Linkage in Cerebrosides.

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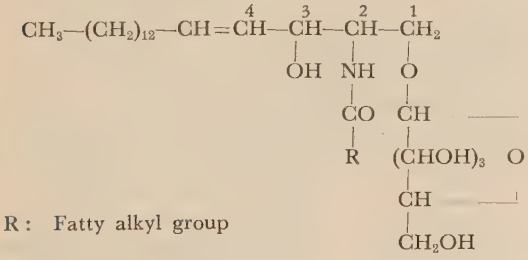
Received June 12, 1956

Enzymatic hydrolysis was executed in order to decide the nature of galactoside linkage of cerebrosides. As a result, it was observed that the β-galactosidase could hydrolyze cerebrosides to liberate the galactose but not so in the case of α-galactosidase. This fact presents one of the conclusive evidences that cerebrosides possess the β-configuration of galactoside linkage in their molecule.

Cerebrosides, which were first isolated from the brain by Thudicum in 1874, are known to be a group of sphingolipids containing the organic base sphingosine in their molecules, the chemical structure of which was well established by Carter and his co-worker[1] as follows:



R: Fatty alkyl group

Methods of stereochemistry which were thereafter applied to the cerebrosides indicate that the double bond and the amino carbon atom of the sphingosine moiety assume the *trans* configuration[2,3] and the D-configuration[4,5] respectively, also that the configuration of carbon atom number three of sphingosine in the state as it exists in the cerebroside molecule is *erythro*-form[6].

With regard to the nature of galactoside linkage in the cerebroside molecule, the results of studies by different authors have led to different conclusions. One view was published in 1950 by Hamasato[7] who insisted on a β-galactoside of psychosine (galactosidosphingosine) on the basis of digestion experiments of this compound by snail liver enzyme, isolating the galactose as methylphenylhydrazone from the digestate. On the contrary, Kiss and his co-worker[8] reported recently in a short communication that the cerebrosides would have the α-linkage of galactose based upon the velocity of hydrolysis, mercaptolytic studies and enzymatic hydrolysis, though the details are not yet published.

Thus, it must be admitted that the problem is still unclear whether the configuration of the galactoside linkage in cerebrosides is either α-form or β-form. In order to approach this problem it seemed to be the most appropriate to investigate the behavior of enzyme towards

* Preceding communications under the same title: Part I, *J. Biochem., Japan*, **39**, 45 (1952); Part II, *ibid.*, **39**, 55 (1952); Part III, *ibid.*, **40**, 251 (1953); Part IV, *J. Biol. Chem.*, in press.

1) H.E. Carter and F.L. Greenwood, *J. Biol. Chem.*, **199**, 283 (1952).

2) K. Mislow, *J. Am. Chem. Soc.*, **74**, 5155 (1953).

3) G. Fodor and J. Kiss, *Nature*, **171**, 651 (1953).

4) H.E. Carter and C.G. Humiston, *J. Biol. Chem.*, **191**, 727 (1951).

5) J. Kiss, G. Fodor and O. Banfi, *Helv. Chim. Acta*, **37**, 1471 (1954).

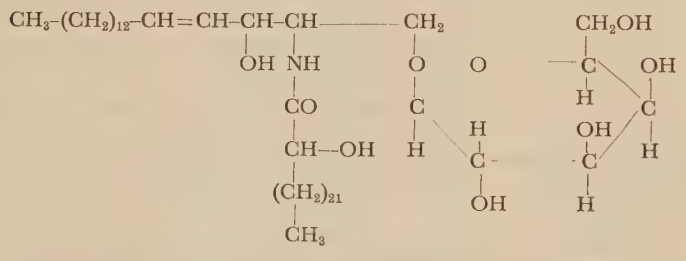
6) Y. Fujino and H.E. Carter, IInd International Conference on Biochemical Problems of Lipids, Ghent, Belgium (1955).

7) Y. Hamasato, *Tohoku J. Exp. Med.*, **53**, 35 (1950).

8) J. Kiss and I. Jurcsik, *Acta Chim. Hung.*, **5**, 477 (1955).

the galactoside linkage of cerebrosides as the previous investigators had undertaken. For this purpose, psychosine sulfate was prepared on one hand from the degradation of phrenosine which is a representative cerebroside, since the psychosine sulfate is much more stable to maintain a colloidal state as a substrate solution than the cerebrosides. On the other hand, α-, and β-galactosidase were separated from the commercial digestive enzyme preparations, and both of them were employed for hydrolysis of psychosine. It was observed that only the β-galactosidase could break down the galactoside linkage of this compound, while α-galactosidase could not. Furthermore, phrenosine and some other cerebrosides were also found to be hydrolyzed by β-galactosidase to liberate the galactose. These results offer almost conclusive evidence that the galactoside linkage takes the form of β-configuration in the cerebrosides molecules.

Upon the basis of facts obtained in the present work along with other data accumulated by investigators so far, the structural formula of the cerebroside would be pictured and characterized as follows, if, for instance, phrenosine is cited:



1-β-galactopyranosyl-2-[α-hydroxytetracosanoyl]-D-*erythro*-2-amino-1,3-dihydroxy-4-*trans*-octadecene.

EXPERIMENTAL

1. Isolation of Phrenosine*. Phrenosine was prepared from a crude sphingolipid fraction of spinal cord. The sphingolipids were dissolved in ten volumes of hot glacial acetic acid; the solution was allowed to stand over night at room temperature, then filtered by suction; the solid was washed with acetone and dried in a desiccator connected to an aspirator. The dried material was fractionated once more in the same way. The treatment of 500 g of sphingolipids yielded 200 g of crude cerebrosides. Chromatography of the latter was run over alumina to remove the phosphorus-containing lipids still remaining. For this purpose, 5 parts of alumina were used effectively for each one part of crude cerebrosides dissolved in 40 parts (by volume) of pyridine. The eluate was distilled under reduced pressure to a small amount and the cerebrosides were precipitated by adding acetone. In this way, 120 g of purified cerebrosides, absolutely free from phosphorus, were obtained from 200 g of crude cerebrosides. As the purified cerebrosides were slightly yellowish and contained some amounts of kerasine, recrystallization was repeated four times from 50 volumes of chloroform-methanol (1 : 1) with charcoal. Phrenosine, thus obtained in a yield of 90 g, was a snow-white crystalline material which was essentially free of kerasine according to the selenite plate test. *Analysis.* Found: N, 1.75%; Calcd. for $C_{48}H_{93}NO_9$ (M. W. 828.2): N, 1.69%. $[\alpha]_D^{20} = +4.5°$ (0.2 g in 10 ml of pyridine).

2. Preparation of Psychosine Sulfate*. Eight g of phrenosine were dissolved in 80 ml of warm dioxane, to which 80 ml of 10% baryta water was added carefully so as to avoid excessive foaming. The reaction mixture was refluxed for ten hours with occasional shaking in order to shake down the adhering material from the inside of the round-bottom flask. The hot yellow-colored solution was poured into 500 ml of water and allowed to stand over night. The precipitate was filtered by suction and washed with water. Then the solid was dried to a constant weight of 9.2 g. The dry solid was ground into a fine powder and was extracted with three 100 ml portions of boiling ethanol. The ethanolic solutions were allowed to cool to room temperature and then filtered by suction. The 300 ml of ethanolic filtrate thus obtained

* These experiments were carried out in the Division of Biochemistry, University of Illinois, Urbana, Illinois, U.S.A. by the senior author, who communicated details on July 28, 1955, at the IInd International Conference of Biochemical Problems of Lipids. Ghent, Belgium.

TABLE I
HYDROLYSIS OF PSYCHOSINE SULFATE WITH DIGESTIVE ENZYMES

Activity	Liberated Galactose mg/ml				
Enzyme	Diastase	Pancreatin	Pepsin-a	Pepsin-b	Trypsin
2 Hrs.	0.00	0.40	0.00	0.36	0.33
4 Hrs.	0.08	0.70	0.14	0.75	0.68

was concentrated under reduced pressure so as to give crude psychosine in a yield of 4.23 g.

The free base, being unsuitable for substrate because of its insolubility in water, was derived to a sulfate, which is easily soluble in water with a tendency of foaming. That is to say, the crude psychosine was dissolved in about 80 ml of ethanol, and precipitated as a sulfate by dropwise addition of 1 N ethanolic sulfuric acid until Congo red paper turned slightly blue. To this solution 80 ml of ether was added to complete the precipitation of sulfate, which was filtered and recrystallized from 1 l of absolute ethanol. Psychosine sulfate was obtained as a beautiful white crystalline material in a yield of 3.5 g.

Analysis. Found: N, 2.70%; Calcd. for $C_{24}H_{47}NO_7 \cdot 1/2\ H_2SO_4$ (M. W. 510.6): N, 2.74%. $[\alpha]_D^{20} = -15.8°$ (0.7197 g in 10 ml of pyridine)

3. Determination of Enzymatic Activity. Enzymatic hydrolysis was carried out in a reaction mixture composed of substrate solution, enzyme solution, methionine solution (activator) and buffer solution. The proportion of the components is stated in the following experiments. After having been adjusted to the proper pH, the reaction mixture was held at 37° in an incubator. An aliquot of the mixture was taken out at definite intervals and subjected to estimation of the liberated galactose by Somogyi's colorimetric method[9].

The control experiment was done by using the substrate and enzyme solution respectively, in parallel with the main experiment. No decomposition was observed in the control test of substrate only, whereas reducing power appeared to some extent only in that of the enzyme. These reducing values of the enzyme were therefore subtracted from the main values of the enzyme-substrate mixture. Thus the degrees of enzymatic activity were represented by the increased amount of the galactose liberated in the hydrolysis of the reaction mixture throughout the experiments.

4. Hydrolysis of Psychosine Sulfate with Commercial Digestive Enzyme Preparations. Galactosidase activity of commercial digestive enzyme preparations (diastase, pancreatin, pepsin-a*, pepsin-b*, and trypsin) towards the psychosine sulfate was tested. Composition of the reaction mixture was as follows: 1% solution of psychosine sulfate–3 parts, 1% solution of enzyme–3 parts, 0.01 M solution of methionine–1 part and buffer solution of glycine-hydrochloric acid–3 parts. The mixture was adjusted to pH 4.0 and incubated at 37° for hydrolysis. If hydrolysis were complete, 1 ml of the reaction mixture would ultimately contain 1 mg of the liberated galactose by calculation. As shown in Table I, diastase and pepsin-a hydrolyzed psychosine sulfate in a very small amount, whereas pancreatin, pepsin-b and trypsin caused hydrolysis of psychosine sulfate to liberate the galactose to the degree of 70%, 75% and 68%, respectively.

5. α-, and β-Galactosidase Activity of Enzymes. As for the digestive enzymes which could hydrolyze psychosine sulfate in the experiment above, activity of α-, and β-galactosidase was examined. Melibiose

TABLE II
α-, AND β-GALACTOSIDASE ACTIVITY OF PSYCHOSINE SULFATE-HYDROLYZING ENZYMES

Substrate	Activity / Enzyme	Liberated Galactose mg/ml: Pancreatin	Pepsin-b	Trypsin
Melibiose	1.5 Hrs.	0.32	0.16	0.28
	4.0 Hrs.	0.50	0.30	0.38
Lactose	1.5 Hrs.	0.54	0.58	0.34
	4.0 Hrs.	0.72	0.75	0.62

9) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).

* Each preparation differs.

TABLE III

SEPARATION OF α-, AND β-GALACTOSIDASE

Substrate	Activity / Enzyme	Liberated Galactose mg/ml					
		Pancreatin		Pepsin-b		Trypsin	
		α	β	α	β	α	β
Melibiose	2 Hrs.	0.08	0.08	0.11	0.00	0.28	0.09
	4 Hrs.	0.11	0.11	0.13	0.00	0.36	0.16
Lactose	2 Hrs.	0.05	0.06	0.05	0.26	0.03	0.15
	4 Hrs.	0.06	0.13	0.08	0.38	0.00	0.21

α: α-Galactosidase β: β-Galactosidase

TABLE IV

HYDROLYSIS OF CEREBROSIDES WITH α-, AND β-GALACTOSIDASE

Enzyme	Activity / Substrate	Liberated Galactose mg/ml			
		Psychosine Sulfate	Phrenosine	Phrenosine Sulfate	Ganglioside
α-Galactosidase	2 Hrs.	0.00	0.06	0.08	0.06
	4 Hrs.	0.00	0.06	0.06	0.10
β-Galactosidase	2 Hrs.	0.20	0.16	0.08	0.24
	4 Hrs.	0.48	0.28	0.20	0.32

was adopted as the substrate for α-galactosidase activity and lactose for β-galactosidase activity. The reaction mixture had the following composition: 1% solution of melibiose or lactose–2 parts, 1% solution of enzyme–2 parts, 0.01 M solution of methionine–1 part and buffer solution of glycine-hydrochloric acid–5 parts. The mixture was put into incubation at 37° and hydrolysis was carried out at pH 4.0. If hydrolysis were complete, 1 ml of this reaction mixture would have to liberate 1 mg of galactose, theoretically.

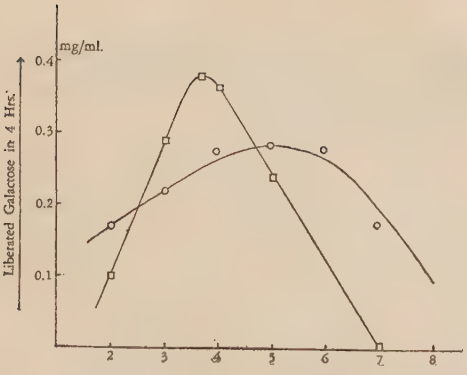
As illustrated in Table II, it was observed that three of the enzymes possessed both α-, and β-galactosidase activity, and also that in each case the latter was more active than the former.

6. Separation of α-, and β-Galactosidase. Separation of α-, and β-galactosidase was attempted according to a modified procedure of kaolin chromatography[10]. For this end, 1 part of the enzyme was dissolved in 100 parts (by volume) of glycine-hydrochloric acid buffer of pH 3.6, and then shaken with 10 parts of kaolin for 30 minutes and centrifuged. The same procedure was repeated twice for the supernatant liquid. The last supernatant fluid was filtered and stored with a small amount of toluene in an ice chest as α-galactosidase solution. The residues from the successive centrifugations were combined and washed four times with 50 parts (by volume) of glycine-hydrochloric acid buffer of pH 5.0, each time with subsequent centrifugation. The centrifugate was shaken vigorously with the latter buffer solution of pH 5.0 for 30 minutes, then centrifuged. The supernatant fluid was filtered and stored with toluene in an ice chest as β-galactosidase solution. Both the α-, and the β-galactosidase solutions lost their activity when stored over five days.

The enzyme solutions thus separated were examined for their action upon the substrate of melibiose and lactose to ascertain whether separation had been sufficiently attained or not. This examination was carried out in the reaction mixture composed of the same proportion of components as described above in Experimental 5. As shown in Table III, the enzymatic activities decreased through all the cases examined, and the separation was not found to have been satisfactorily accomplished in most cases. It was, however, found that α-galactosidase without the β-one was separated from the trypsin preparation and β-galactosidase without the opposite from the pepsis-b preparation respectively, though both in lower grades of activity.

7. Optimum pH of α-, and β-Galactosidase. The glycine-hydrochloric acid buffer was prepared for the range of pH 2.0–4.8 and the phosphate buffer for pH 5.0–8.0. The composition of the reaction mixture was the same as in Experimental 5. The figure illustrates the pH curve of α-galactosidase from trypsin and that of β-galactosidase from pepsin-b. As shown, the optimum pH of α-galactosidase was

10) Nagaoka, *Tohoku J. Exp. Med.*, **51**, 137 (1949).



Optimum pH of α-, and β-Galactosidase
○ α-Galactosidase activity
□ β-Galactosidase activity

not sharp, that is, an optimal zone was found between pH 4.0 and 6.0, whereas, β-galactosidase showed a considerably sharp activity at pH 3.6–3.7. The results obtained here agreed rather well with those of previous investigators[11].

8. Hydrolysis of Cerebrosides with α-, and β-Galactosidase. In studying the behavior of α-, and β-galactosidase towards the cerebrosides, the authors examined not only phrenosine but also phrenosine sulfate* and ganglioside* as substrates. The latter substances were found to be more soluble in water than the former in making a homogeneous colloidal solution. To the reaction mixture the enzyme solutions were added in such an amount that galactosidases, of which the activity had decreased greatly after separation, would attack the substrates as actively as possible. The reaction mixture for this experiment had the following composition: 1.0–1.5% solution of the cerebrosides–3 parts, enzyme solution–13 parts, 0.01 M solution of methionine–1 part and buffer solution of glycine-hydrochloric acid–3 parts. The incubation mixture was adjusted to pH 5.0 for the α-galactosidase and to pH 3.6 for the β-galactosidase, in accordance with the results described above in Experimental 7. In case that the reaction mixture contained phrenosine or psychosine sulfate as the substrate, 1 ml of the reaction mixture should include almost 0.5 mg of the liberated galactose if complete hydrolysis should occur. As shown in Table IV, hydrolysis was not observed in the experiments with α-galactosidase, whereas the β-galactosidase was found to hydrolyze all the substrates examined, resulting liberation of the galactose in a fairly good percentage.

The authors wish to express their sincere thanks to Prof. Yukihiko Nakamura, Dept. of Agricultural Chemistry, Hokkaido University, for his kind advice and suggestions.

11) *e. g.*, S. Veibel and G. Oestrup, *Biochim. et Biophys. Acta*, **1**, 1 (1947).

* The authors are indebted to Mr.E. Okuhara, Department of Medical Chemistry, Hokkaido University for preparation of these compounds.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 188~196, 1956]

Studies on the Bacterial α-Amylase, Especially in Regard to the Role of Calcium Contained

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The role of calcium contained in crystalline bacterial α-amylase is observed, in part, from the effects on proteochemical properties of the enzyme caused by removal of the metal. The deprivation of calcium from the enzyme results, in fact, not only in loss of activity, but also in denaturation and change in electrophoretical properties of the enzyme protein. It indicates that the metal plays an important role just like a rivet on a fan to retain the native state as well as the activity of the enzyme.

It has long been known that calcium salts remarkably increase the stability of α-amylase to heat and acids. Recent works, involving the success in crystallization of α-amylases from several sources,[1-6] have revealed that the crystalline amylase contains calcium as an inherent constituent, and that removal of this metal results in loss of enzme activity. It has also been found that, in the crystallization procedure of α-amylase more amount of calcium than that for the maintenance of activity of the enzyme is required. Hence, the whole metal contained in the crystalline enzyme seems not always to be necessary to maintain the activity.[4,7,8]

It has been reported that addition of calcium salts to bacterial α-amylase brings forth a suppressive effect on saccharifying activity without having any effect on dextrinization.[7,9,10] Hitherto, however, the calcium metal which plays an important role in α-amylase has not yet received so much attention as heavy metals such as iron, copper, manganese and cobalt in other enzymes.

Recently, the author has reported that an α-amylase-destroying enzyme attacks only those α-amylases containing calcium, and hence, suggested that the attack point of the destroying enzyme might be situated on the calcium-linking part of those α-amylase protein.[11] In the present paper, in order to obtain further knowledge of the significance of calcium in α-amylase, the experiments on the deactivation of bacterial α-amylase by treatment with such calcium-removing agents as ethylenediamine tetraacetate (EDTA) and oxalates and observations on the properties of amylase protein thereby deactivated, were attempted.

MATERIALS AND METHODS

Bacterial α-amylase used here was recrystallized until it was entirely liberated from contaminated protease detectable by the following method. To the amylase solution, ammonium oxalate was added to

1) S. Schwimmer and A.K. Balls, *J. Biol. Chem.*, **179**, 1063 (1949).
2) Ed. H. Fischer et C.H. Haselbach, *Helv. Chim. Acta*, **34**, 325 (1951).
3) B. Hagiwara, *Symposia on Enz. Chem. (Japan)*, **7**, 105 (1952).
4) S. Akabori, T. Ikenaka and B. Hagiwara, *ibid*, **7**, 107 (1952).
5) M.L. Caldwell, M. Adams, J. Tung and C.C. Toralbala, *J. Am. Chem. Soc.*, **74**, 4033 (1952).
6) V.M. Hanrahan and M.L. Caldwell, *ibid*, **75**, 4030 (1953).
7) T. Yamamoto and J. Fukumoto, *Symposia on Enz. Chem. Japan*, **10**, 32 (1954).
8) S. Akabori, T. Ikenaka, J. Oikawa, H. Hanabusa, H, Tsugita and T. Matsushima, Lecture delivered at the Symposia on Enz. Chem., Osaka, Japan, July 17, 1955.
9) J. Fukumoto, T. Yamamoto and A. Kasugai, Lecture delivered at the Meeting of Agr. Chem. Soc., Kyoto, Japan, Sept. 22, 1951.
10) S. Ono and K. Hiromi, *Proc. Jap. Acad.*, **30**, 467 (1954)
11) T. Yamamoto, This Bulletin, **19**, 20 (1954).

make up the concentration to M/30. The mixture was left at 15°C for two days, the enzyme completely losing its activity thereby. Then, TCA-acetic-acetate mixture, composed of 0.33 M CCl_3COOH, 1.0 M CH_3COOH and 0.67 M CH_3COONa, was added. To the filtrate was applied Folin's phenol reagent[12]. By employing this method it was possible to detect such a trace of protease that could not be found by usual methods with casein or gelatin as the substrate.

For the kinetic study of deactivation of the enzyme with EDTA or oxalates, no buffer solution was used except for the experiment on the effect of pH in the presence of oxalate. Determination of EDTA remaining after the reaction was done by a titration method with calcium acetate solution, using Eriochrome Black T as the indicator[13]. Amylase activity was assayed by electrophotometry that determins the activity to reduce the blue color value of soluble starch, developed by iodine. The crystalline amylase used here, gave 13.100 units per mg nitrogen on this assay method[14]. In the investigation of digestibility by protease of the deactivated amylase, the solution of crystalline protease of *Bacillus subtilis* was added to the amylase solution, followed by the addition of calcium-removing agent. Aliquots of the reaction mixture were taken, at some intervals, in to an equal volume of TCA-acetic-acetate mixture, and this was filtered after standing for thirty minutes at 40°C. A modification of the Folin-Ciocalteau's method[15] was applied to the filtrate and color value compared with that of the standard digests of amylase, where the

12) O. Folin and V. Ciocalteau, *J. Biol. Chem.*, **73**, 627 (1927).
13) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds", Printice Hall, New York, 1952, p. 427

14) The activity units expressed by the method almost agreed with those given by the author's old method in determination of starch liquefaction activity (See (7)).
15) T. Yamamoto, This Bulletin, **19**, 121 (1955).

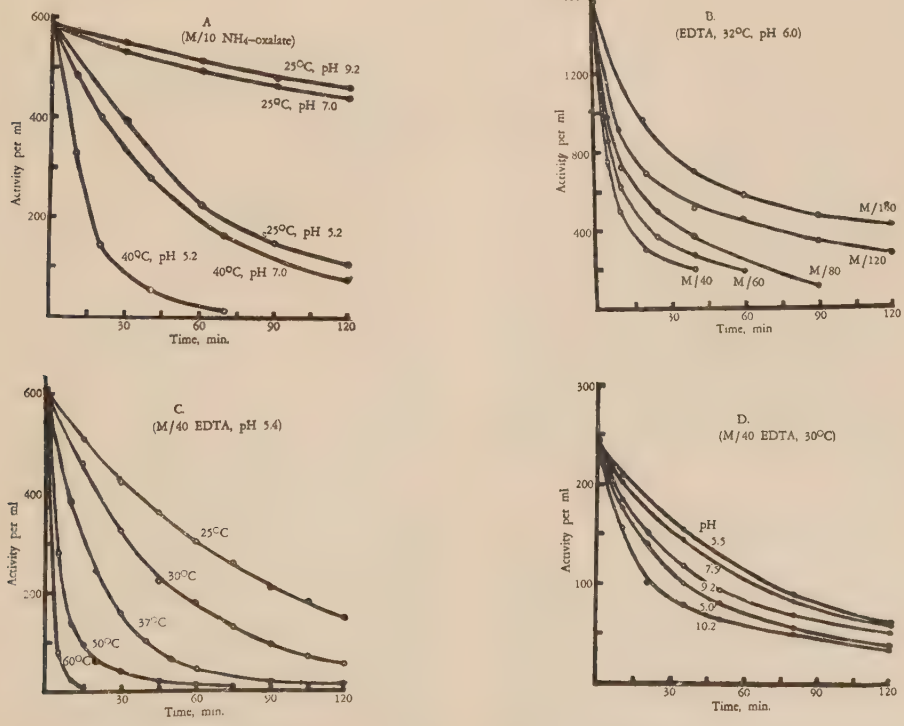


FIG. 1. Deactivation of Bacterial α-Amylase by NH_4-Oxalate (A), by EDTA as the Function of the Concentration (B), Temperature (C) and pH (D).

In (A) M/40 acetic-acetate, M/120 phosphate, or M/40 NH_4OH-NH_4Cl buffer at the final concentration was added. In (B), (C) and (D) disodium-EDTA was neutralized with NaOH respectively to the pHs noted on.

TABLE I

DEACTIVATION OF BACTERIAL α-AMYLASE BY EDTA AND CONSUMPTION OF THE REAGENT

(Initial amylase activity of the reaction mixture, 6.300 u/ml; 37°C, pH 9.0*)

EDTA given, μ mol/ml	2.0			1.0			0.5		
Time, min.	28	85	330	28	85	330	28	85	330
Amylase deactivated, u/ml	1880	3540	6124	1700	3320	6102	1400	3150	6050
EDTA consumed, μ mol/ml	0.132	0.165	0.215	0.084	0.121	0.155	0.054	0.091	0.102

* Disodium EDTA was neutralized with NH_4OH to 9.0 and added to the amylase which had been dissolved with dil. NH_4OH and adjusted to pH 9.0. Determination of the EDTA was carried out at pH 9.8 by the presence of M/50 NH_4OH.

TABLE II

COMPARISON OF DEACTIVATION POWER OF THE AMMONIUM AND SODIUM SALT OF CALCIUM REMOVING AGENTS

(Final conc. of the reagents, M/10; initial amylase activity of the reaction mixture, 120 u/ml; 40°C, pH 7.0, 60 min.)

	EDTA		Oxalic acid		Citric acid	
Cation	NH_4^+*	Na^+	NH_4^+	Na^+	NH_4^+	Na^+
Remaining amylase Activity, u/ml	5.0	15.0	9.5	65.0	12.0	71.0

* Disodium EDTA was neutralized with NH_4OH.

same amount of amylase as in the test run was denatured with 0.2 N sodium hydroxide which, after neutralization, was digested by the bacterial protease.

As for electrophoretic experiments, the Tiselius electrophoresis apparatus (Hitachi) adopting the cylindrical lens schlieren optical system[16] was used. The sample, with a phosphate buffer of which the concentration was a little higher than the outer solution, was dialysed in a cellophan bag against a large quantity of phosphate buffer ($\Gamma/2$, 0.12) for forty-eight hours at 10°C, thus renewing the outer solution three or four times and stirring the content of the dialysis bag frequently. Electrophoresis was always performed under a current of 11.5 m amp, 84 V and at 3°C, but electric conductivity of the sample was not determined.

EXPERIMENTAL RESULTS

The results of the deactivation test of bacterial α-amylase with EDTA and oxalates as the functions of reagent concentration, temperature and pH, respectively, are shown in Fig. 1, indicating that the deactivation roughly follows the equation of a first-order reaction. However, the deactivation dose not always run parallel to consumption of the calcium remover. Over a certain range of concentration of EDTA, the consumption appears to be almost proportional to the initial concentration of the reagent, while the degree of deactivation is quite independent thereof, as given in Table I. The effect of pH on deactivation was found to appear only in the vicinity of the stability limit of the enzyme (Fig. 1). Deactivation power of calcium-removing agents differs remarkably, according to the nature of their salt and it was found that the ammonium salts are steady and stronger in power than sodiums' ones (Table II). Of a number of organic and inorganic salts examined, concerning their calcium-deprivation effect, only citrate revealed to have a power approximately similar to that of the oxalates while the others were either exceedingly weak (malonates) or entirely negative. Progress of enzyme deactivation by calcium remover can be arrested on the spot by the addition of starch, but, on completion of the hydrolysis of starch, the deactivation is restored in full activity and proceeds almost in the same rate as before (Fig. 2).

The effect of bacterial protease on the deactivated amylase is shown in Fig. 3. This experiment was carried out with oxalate alone, as EDTA, itself, reacts with Folin's phenol reagent, developing a blue color and interfering with the determination of the digested protein. Fig. 3 shows that the amylase protein turns protease-digestible as soon as it is deactivated by oxalate. This also seems to be the case with EDTA-

16) L. G. Longsworth, *Ind. Eng. Chem., Anal. Ed.*, **18**, 219 (1946).

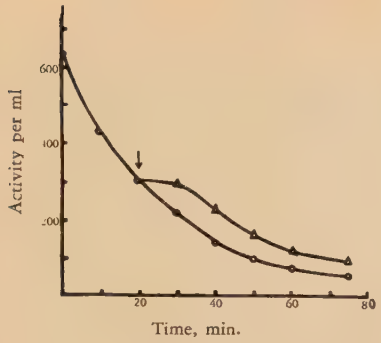


FIG. 2. Effect of Starch on the Amylase being Deactivated with Oxalate (NH_4-oxalate, M/6; 40°C, pH 6.8).

At the arrow point 1 ml of 10 % soluble starch was added to 4.5 ml of the reaction mixture.

deactivation, because, it was observed that in the presence of bacterial protease, the precipitate formed by TCA-acetic-acetate mixture decreases in amount with the progress of deactivation.

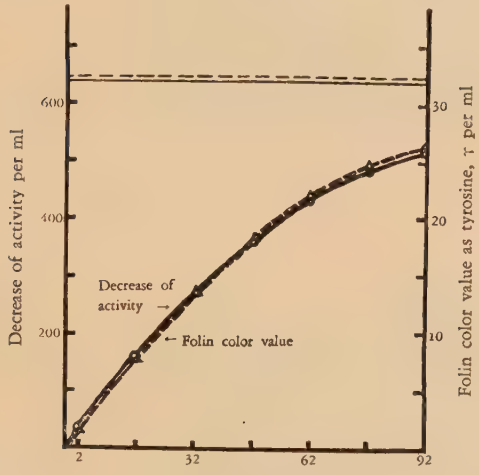
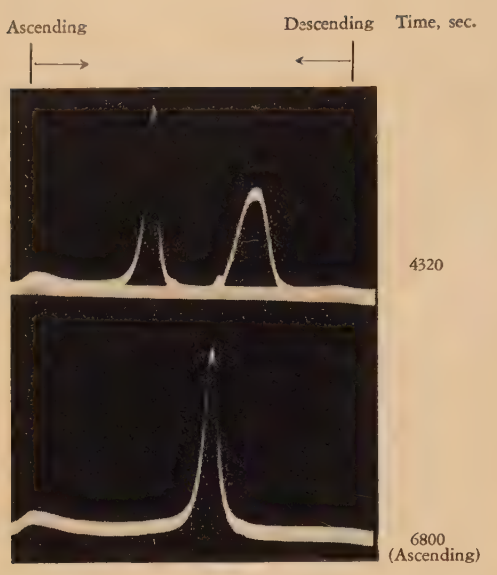


FIG. 3. Deactivation of Bacterial α-Amylase with NH_4-Oxalate and Digestibility of the Deactivated Amylase by Bacterial Protease.

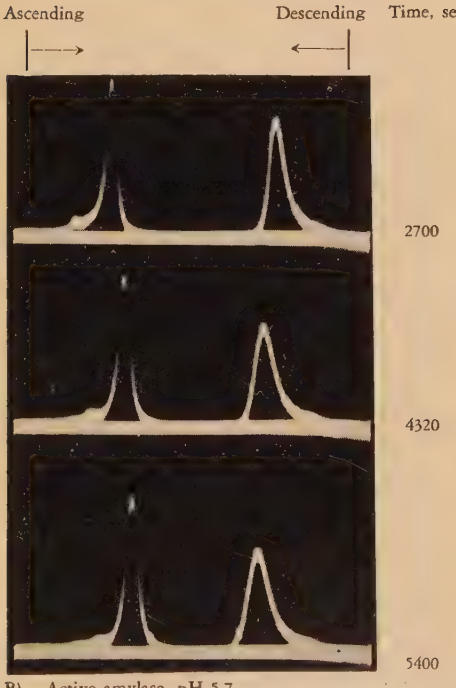
(6 ml of M/3 NH_4-oxalate was added to 10ml of the mixture of amylase (5.3mg) and protease (0.04mg) and kept at 40°C, pH 6.6). The upper solid line and broken line indicate the limit of the activity to be lost and the full Folin color value of the reaction mixture, respectively.

Photographs of the electrophoretic pattern of active bacterial α-amylase and the amylase protein deactivated with EDTA, are shown in Figs. 4, 5 & 6. As a higher concentration of the enzyme protein is necessary for experiment of this kind, only EDTA was found useful, on the basis that deactivation with EDTA produces only a slight opacity in the solution, even in a considerably higher concentration of the enzyme protein, while, in the case with oxalate, a precipitate too thick, is formed in such high concentration. The crystalline bacterial α-amylase suspended in distilled water was dissolved with the least amount of N/20 sodium hydroxide up to the protein concentration of 2 to 3% with supplemental distilled water. After neutralizing with acetic acid to pH 7.0, EDTA (pH 7.0) was added thereto so as to make the concentration up to M/120 (sometimes, M/60 or M/240) and the mixture was kept at 50°C. Then, the enzyme completely lost its activity within two or three hours, while viscosity of the solution increased remarkably. Electrophoresis was performed with both samples which had lost activity wholly and partially (Figs. 4, D & 6). The former sample was also examined after mixing with active amylase (Fig. 4, C). In this case, the deactivated amylase solution, which had been dialysed against distilled water for fourteen hours, was mixed with an active amylase solution in an equal amount of enzyme protein and dialysed again, as above. A small quantity of precipitate and crystals, which had been formed during dialysis, were removed by centrifugation and the supernatant solution was used for the electrophoretic experiment. For comparison, electrophresis was also performed with the sample denatured by hydrochloric acid (Fig. 5). To the active enzyme solution, N/5 hydrochloric acid was added to bring the pH down to 1.6 and the solution, being stood for thirty minutes at 10°C (The enzyme solution containing either little or no salt remains clear on heating or acidifying.), was neutralized with N/5 sodium hydroxide solution up to pH 5.8~6.0, where the denatured enzyme protein coagulated into curd. Then, N/20 sodium hydroxide was added to it with stirring, and, after dissolving (pH 8.6), dialysed.

As it will be seen in the photographs, the electrophoretic pattern of EDTA-deactivated amylase is essentially different from that of the active amylase and has two main boundaries, one, migrating more rapidly than the original amylase protein (a), and the other following close to this (b). The former is major component. Similar results are also found on the enzyme deactivated partially with EDTA and on the hydrochloric acid-denatured one. There will be seen

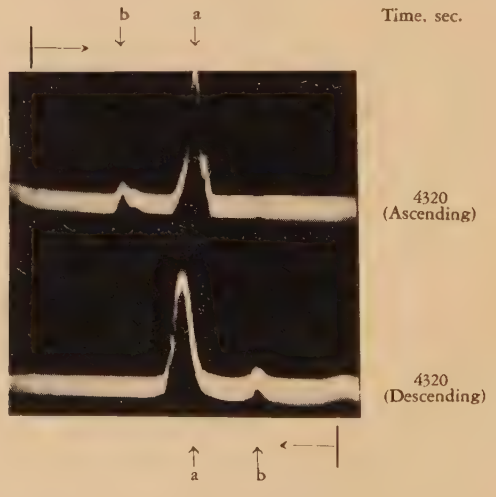


A). Active amylase, pH 7.7.



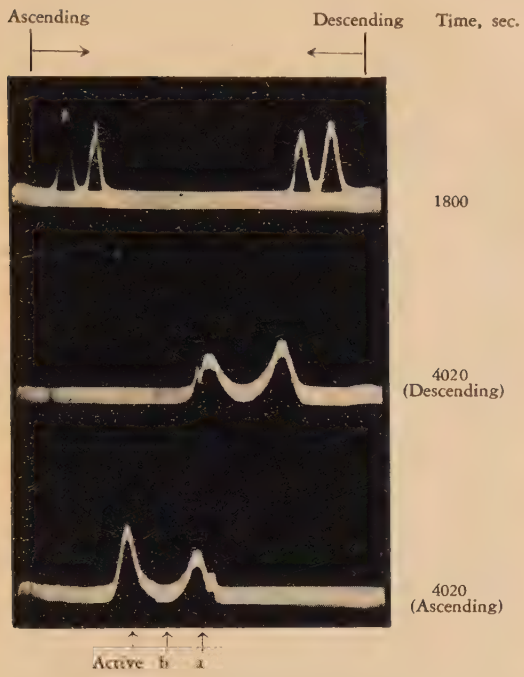
B). Active amylase, pH 5.7.

C). Active amylase plus EDTA-deactivated amylase, pH 7.7.

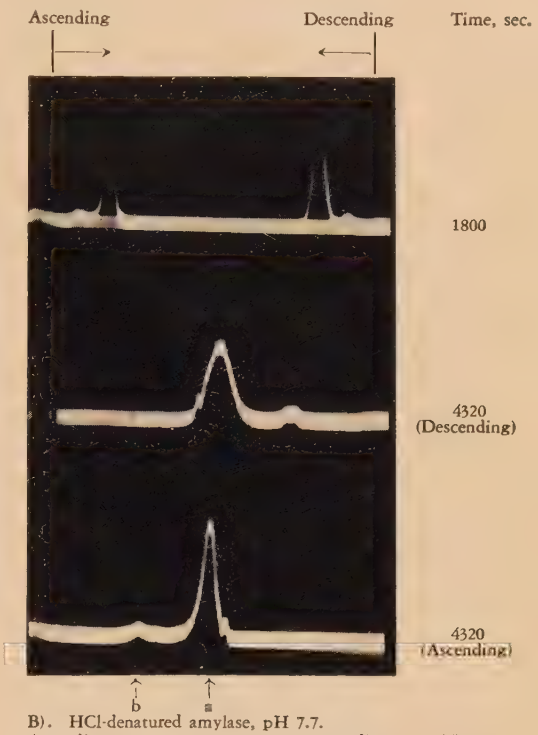


D). EDTA-deactivated amylase, pH 7.7.

FIG. 4. Electrophoretic Patterns of Active Amylase and EDTA-Deactivated Amylase.



A). Active amylase plus HCl-denatured amylase, pH 7.7.



B). HCl-denatured amylase, pH 7.7.

FIG. 5. Electrophoretic Patterns of HCl-denatured Amylase.

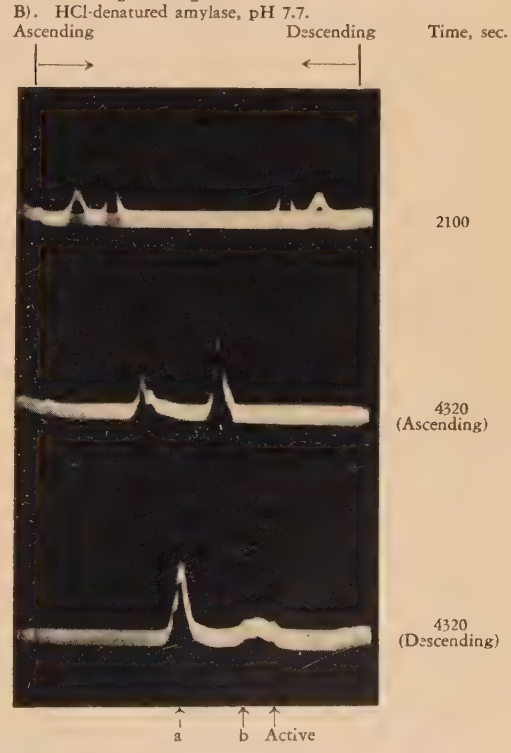


FIG. 6. Electrophoretic Patterns of Amylase Deactivated Partially with EDTA, pH 7.7.

(When about three quarters of the total activity were lost, lead acetate equivalent to EDTA was added, whereby the falling of the activity had been completely stopped, and then dialysed.).

a small but distinct notch on the shoulder in the progressing side of boundary a. However, this was not observed during the moving of the boundaries.

DISCUSSION

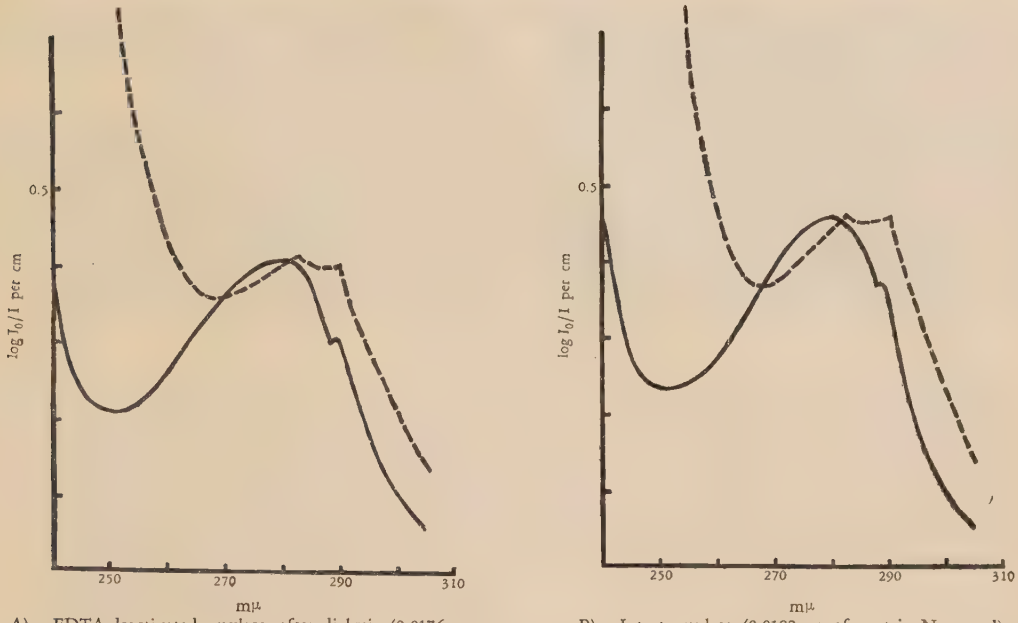
From the experimet on amylase deactivation with EDTA which gave a result that the rate of reagent consumption is greater than that of the enzyme deactivation at an early stage of reaction, it might be supposed that, besides the essential calcium for maintaining activity, an extra calcium will exist in the enzyme which dose not participate in enzymic activity but links to the enzyme in almost the same strength as the essential one, and that removal of the extra metal may cause some minor change in the charge state of the amino acid residues to which the metal had linked, bringing such a modification to the enzyme molecule that it can be protected to some extent, from the deprivation of the essential calcium by the metal catcher. This seems to be of interest in connection with the fact that the taka α-amylase once treated with a small quantity of EDTA shows greater endurability than the untreated one upon addition of a certain amount of the reagent[8].

In the experiment shown in Fig. 3, it was found that the amylase protein deactivated with oxalate, becomes digestible by bacterial protease into a completely soluble state in TCA-acetic-acetate mixture. It has been known that the active amylase cannot be attacked by protease,[11] whilst, the bacterial protease attacks various kinds of denatured proteins, making them soluble quantitatively in trichloroacetic acid solution.[17] Accordingly, the coincidence of the degree of deactivation with that of protease-digestibility of the amylase protein treated with oxalates seems to show that the calcium playing the main role in maintaining activity, also shares a role in protecting the enzyme from denaturation and attack by protease.

It has been known that denaturation of protein, in general, brings about only small changes in electrochemical properties despite the liberation of significant numbers of reactive groups or large alterations in molecular kinetic properties, and that changes in electrophoretic properties, if they occur, are mostly those of which the isoelectric point of the protein displaces toward neutrality.[18] However, as shown in the photographs, EDTA was found to alter the enzyme to protein of a quite different character electrophoretically, where two components are represented, one of which moves toward anode more rapidly than the intact enzyme protein and the other, in almost the same velocity. The former protein, more acidic, seems to be overwhelming in quantity. Similar patterns were also obtained by the denaturation of the enzyme with hydrochloric acid. It is almost unlikely that the change in electrophoretical properties under the above condition may be due to the action of any contaminated protease in the crystalline amylase or protease contaminated during the process of this experiment. For, any decomposition product of the deactivated enzyme protein could not be detected in the dialysate or in the mother liquor after elimination of protein with TCA-acetic-acetate mixture. However, in the ultraviolet absorption spectra measured on an alkaline side, the EDTA-deactivated enzyme solution which had been dialysed against distilled water gave a value somewhat higher in the ratio of the extinction at 268 mμ to that of 282 mμ, when compared with that of the active amylase solution (Fig. 7), although the former showed almost negative reaction to the determination test of EDTA with calcium acetate solution. This might be the indication of a trace amount of EDTA remaining in the dialysed solution, which makes the suggestion possible that the change in electrophoretical properties was due to the interaction of EDTA or EDTA-calcium

17) K. Okunuki, B. Hagiwara et al., *Symposia on Enz. Chem. (Japan)*, **9**, 1 (1954); *ibid*, **10**, 143 (1954).

18) F.W. Putnam, "The Protein", edited by H. Neurath and K. Bailey, 1954, p. 849.



A). EDTA-deactivated amylase, after dialysis (0.0176 mg of protein N per ml).

B). Intact amylase (0.0182 mg of protein N per ml).

FIG. 7. Ultraviolet Absorption of Bacterial α-Amylase.
(Solid line, in distilled water; broken line, in N/10 NaOH).

chelate compounds with the deactivated enzyme protein. However, no particular difference in mobility was found among the enzyme proteins deactivated with EDTA of M/60, M/120, and M/240, respectively. Accordingly, it will be reasonable to conclude that the change in electrophoretical properties must be due to the increase of the carboxylate group in the enzyme protein as a result of the removal of the calcium contained. It might also be possible to uphold this idea in the case of denaturation by hydrochloric acid.

However, no experimental result has yet been obtained that can prove the reason why the protein fragment, which possesses mobility (ies) close to the intact amylase (Fig. 4, D & 5, B) but seems to be a little in quantity, divergently develops in the deactivation process by EDTA and hydrochloric acid. But, the results obtained here lead to the assumption that a difinite amount, ca. one atom, of the calcium in the bacterial α-amylase probably links, on the one hand to carboxyl groups of certain residual or terminal amino acids while, on the other hand, to non-ionic groups of some amino acid residues of the enzyme protein, thereby forming a certain chelate type compound, which is decisively responsible for the native state as well as the activity of the enzyme.

SUMMARY

In order to approach the problem on the role of calcium conatined in crystalline bacterial α-amylase, effects of calcium catchers such as EDTA and oxalates, on the enzyme protein were investigated and the following results were obtained:

1. The calcium catchers deactivate the enzyme while being consumed much rapidly in compare with the extent of its deactivation ability, in the early stage of reaction.

2. The reagents alter the enzyme, causing deactivation, to a protein susceptible to protease.

3. The reagents also provoke a remarkable change in the mobility of the enzyme protein.

4. From these results, it may be concluded that there are two types in the binding mode of calcium in the crystalline enzyme: one, forming a chelate compound and playing a decisive role on the maintenance of activity, native state and electrophoretical properties of the enzyme, and the other, linking without any relation to those properties of the protein, although the removal of the calcium might cause some minor change in the molecular form of the enzyme protein.

The author expresses his sincere thanks to Prof. I. Yamasaki of Kyushu University and Prof. J. Fukumoto of Osaka City University for their encouragement. This work was aided in part by a grant from the Scientific Research Fund, Ministry of Education.

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Separation and Identification of Fatty Acids

Part XX. 2, 4-Dinitrophenylhydrazones of *p*-Bromophenacyl Esters as Derivatives for Characterization of Unsaturated Fatty Acids[1]

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Received June 18, 1956

For the characterization of fatty acids, 2, 4-dinitrophenylhydrazones of *p*-bromophenacyl esters of even-numbered saturated fatty acids from C_2 to C_{20} and several unsaturated fatty acids from monoethenoid to triethenoid were prepared. The derivatives of linoleic and linolenic acids as well as those of the other unsaturated and saturated acids, were successfully obtained in crystalline forms which showed sharp and high melting points, 72° and 69°, respectively. It was found that the derivatives of unsaturated acids were valuable for characterizing the parent acids, while those of saturated acids were unsuitable for this purpose owing to the similarity of their melting points.

The separation and identification of unsaturated fatty acids are usually carried out through the bromination or hydroxylation procedure by converting unsaturated acids into the bromo- or hydroxy-derivatives of their corresponding saturated fatty acids. However, both of these derivatives involve as yet unsolved problem of isomerism and other inevitable drawbacks, which have already been pointed out.[2,3] The derivatives obtained by the reaction of the carboxyl groups of the acids which seemingly have none of such drawbacks, are therefore more suitable for this purpose.

A few soild derivatives of unsaturated fatty acids are reported in the literature. Drake et al.[4] and Kimura[5] prepared *p*-halo- and *p*-phenylphenacyl esters of oleic series. Kass et al.[2], furthermore, prepared the *p*-phenylphenacyl esters and S-benzylthiuronium salts of di- and trienoic acids (m. p., *p*-phenylphenacyl esters, linoleate, 37–37.5°, 46.5–47°; linolenate, 37.5–38°, 38–39°; S-benzylthiuronium salts, linoleate, 123.5–125°; linolenate, 122–123°). Swern et al.[3] also obtained N-(2-hydroxyethyl)- and N-(*n*-dodecyl)-linoleamide (m. p., the former, 39–39.5°; the latter, 45.5–46°), while the hydroxamic acids of linoleic and linolenic acid (m. p., the former, 41–42°; the latter, 37–38°) were prepared by Inouye and Yukawa.[6]

These derivatives of di- and trienoic acids are, however, still disadvatageous for achieving identification of the acids, because of their high instabilities, similarity of melting points, relatively low melting points, and some impurities impossible to remove by recrystallizations (indicated by the disagreement of iodine numbers with the calculated values).

In our present work, 2, 4-dinitrophenylhydrazones of *p*-bromophenacyl esters, as

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1) Presented at the annual meeting of the Japan Oil Chemists' Society, Nagoya, November 8, 1955.

2) J. P. Kass, J. Nichols, and G. O. Burr, *J. Am. Chem. Soc.*, **64**, 1061 (1942).

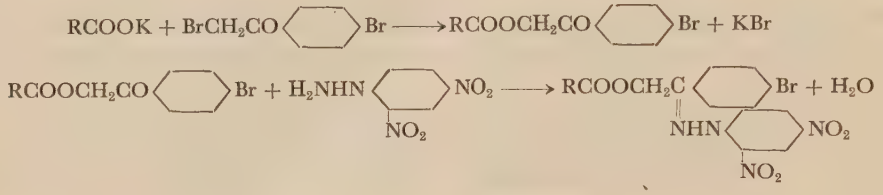
3) D. Swern, J. M. Stutzman, and E. T, Roe, *ibid.*, **71**, 3017 (1949).

4) N. L. Drake and J. Bronitsky, *ibid.*, **52**, 3715 (1930).

5) W. Kimura, *J. Soc. Chem. Ind. Japan*, **35**, 643 (1932).

6) Y. Inouye and H. Yukawa, *J. Agr. Chem. Soc. Japan*, **16**, 510 (1940).

derivatives more valuable for characterization purposes, are prepared in the following manner.



EXPERIMENTAL

Fatty Acids. Arachidic acid was prepared from stearyl alcohol by the malonic acid synthesis. The other even-numbered saturated fatty acids from acetic to stearic were all commercial preparations. 10-Undecenoic acid was obtained by the pyrolysis of castor oil. Oleic, erucic, and ricinoleic acids were prepared from natural sources in the usual manner. Elaidic and stearolic acids were derived from oleic acid, and brassidic and behenolic acids from erucic acid by usual methods. Linoleic and linolenic acids were prepared by the debromination method. All these fatty acids were sufficiently purified, and their high purities were ascertained by determination of their physical and chemical constants.

PROPERTIES AND ANALYSES OF 2, 4-DINITROPHENYLHYDRAZONES OF *p*-BROMOPHENACYL ESTERS OF FATTY ACIDS

Acid	m.p., °C.	N, % Calcd.	N, % Found	I. V. Calcd.	I. V. Found
Acetic	182	12.82	12.63	...	...
Butyric	143	12.04	11.91	...	...
Caproic	115.5	11.36	11.40	...	...
Caprylic	108.5	10.75	10.68	...	...
Capric	111.5	10.20	10.01	...	...
Lauric	110.5	9.70	9.82	...	...
Myristic	110.5	9.25	9.09	...	...
Palmitic	110.5	8.84	8.98	...	...
Stearic	111	8.47	8.35	...	...
Arachidic	111	8.12	8.03	...	...
10-Undecenoic	103	9.98	9.79	45.2	44.5
Oleic	76.5	8.49	8.56	38.5	38.1
Erucic	78.5	7.83	7.71	35.5	35.1
Linoleic	72	8.52	8.41	77.2	76.6
Linolenic	69	8.55	8.60	116.1	116.0
Elaidic	93	8.49	8.42	38.5	38.0
Brassidic	96	7.83	7.78	35.5	34.3
Stearolic	71.5	8.52	8.61	38.6*	39.4
Behenolic	72.5	7.85	7.78	35.6*	35.9
Ricinoleic	61	8.29	8.14	37.6	36.7

*Calculated as one mole of iodine added to a triple bond.

Preparation. A solution of fatty acid in 95% alcohol was neutralized with 0.5 N alcoholic potassium hydroxide until the solution became just neutral or slightly acidic. The neutralized solution was refluxed for one hour with an amount of *p*-bromophenacyl bromide slightly smaller than the theoretical. The mixture was cooled to 20—30° (0° in the cases of unsaturated acids), and poured dropwise into a slightly excess of 2, 4-dinitrophenylhydrazine in 2 N methanolic hydrochloric acid, under stirring. After standing for two to three hours at 0° and then diluting with a small volume of water, the product was collected by filtration and freed from hydrochloric acid by washing repeatedly with cold water. Two or three recrystallizations from absolute alcohol gave yellow crystals of the pure 2, 4-dinitrophenylhdyrazone derivatives in a high yield (80—90%). The derivatives of linoleic and linolenic acids were treated under nitrogen throughout the processes of their preparation. The melting points, iodine values, and analyses of nitrogen of the derivatives thus obtained are summarized in the accompanying Table.

RESULTS AND DISCUSSION

As shown in the Table, it was found that all of the *p*-bromophenacyl ester 2, 4-dinitrophenylhydrazones of fatty acids showed sufficient high melting points and stabilities enabling them to be prepared in pure crystalline state. Though these hydrazone derivatives may be attached to a disadvantageous problem of the *syn-* and *anti-* isomers, as expected from recent investigations[7-12], yet

7) H. H. Szmant and H. J. Planinsek, *J. Am. Chem. Soc.*, **72**, 4042 (1950).
8) H. B. Stewart, *Biochem. J.*, **55**, xxvi (1953).
9) D. Cavallini and N. Frontali, *Biochim. Biophys. Acta*, **13**, 439 (1954).
10) H. van Duin, *Rec. trav. chim.*, **73**, 78 (1954).
11) F. A. Isherwood and D. H. Cruickshank, *Nature*, **173**, 121 (1954).
12) F. A. Isherwood and R. L. Johnes, *ibid.*, **175**, 419 (1955).

it seems that the conditions used in the above preparation result in only one isomer of the derivatives in high yield, and even if the other isomer was slightly produced in the reaction mixture, it may be easily removed by recrystallization of the products. This view is supported by the considerably sharp melting points observed in all the derivatives of saturated and unsaturated fatty acids.

It also appears that both the double and triple bonds of unsaturated fatty acids in the derivatives were not affected during the processes of their preparation, as indicated by the iodine values which agreed with the theoretical.

The melting points of the derivatives of higher saturated fatty acids showed close similarity with those of the adjacent members. However, the derivatives of unsaturated fatty acids demonstrated considerable differences in melting points between individual compounds except those of stearolic and behenolic acids, thus, these derivatives may be recommended as a usable derivative for the identification of unsaturated acids, particularly linoleic and linolenic acids.

The *p*-bromophenacyl ester 2, 4-dinitrophenylhydrazones of fatty acids were generally very soluble in benzene, and slightly soluble in petroleum ether. An investigation on the utilization of the derivatives to effect the separation of fatty acids, has indicated that in many organic solvents the solubilities of the derivatives are too similar to accomplish this purpose.

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Separation and Identification of Fatty Acids

Part XXI. Paper Chromatography of Fatty Acids as Their *p*-Bromophenacyl Ester Derivatives[1]

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Received June 18, 1956

By reversed-phase paper chromatography, the simultaneous separations of all saturated and unsaturated fatty acids were successfully carried out on the same paper with the aid of *p*-bromophenacyl ester 2, 4-dinitrophenylhydrazones and their mercuric acetate addition compounds. When petroleum hydrocarbon (b. p. 140—170°) was used as the stationary solvent and methanol—acetic acid—petroleum hydrocarbon as the moving solvent, even saturated acids from C_4 to C_{22}, even monoethenoid acids from C_{10} to C_{22}, and C_{18} series from stearic to linolenic were well separated, respectively. Similar separations were also attained on paper impregnated with decalin and olive oil. From the results of several applications, this method has been found to be effective for the analysis of the component acids in natural fats.

A few principles of paper chromatography for simultaneous separations of both higher saturated and unsaturated fatty acids have been suggested by several workers.[2-4] However, each of these techniques is available for only a small range of fatty acids, being insufficient in the separability of spots for practical use.

A satisfactory separation of the mixed fatty acids has been shown by paper chromatography methods using fatty acid derivatives, i. e., 2, 4-dinitrophenylhydrazides,[5] acetol ester 2, 4-dinitrophenylhydrazones,[6] mercuric acetate addition compounds,[7] when only saturated or unsaturated fatty acids are analyzed. In the present work, an effort was further made to develop an improved method in which the total analysis of saturated and unsaturated fatty acids is capable on the same chromatogram, by the combining use of *p*-bromophenacyl ester 2, 4-dinitrophenylhydrazones and their mercuric acetate addition compounds. In the present method, all fatty acids are directly detected as yellow spots, and then the unsaturated acids are selectively identified as green spots by spraying with a solution of diphenylcarbazone. Although the spots of lower saturated acids interfere with those of some unsaturated acids, these difficulties are considerably eliminated when the identification is performed by combining the separation of the hydrazone mixture with that of their mercurated products.

The technique of chromatographic separation of the fatty acid derivatives and its several applications to the analysis of the component acids in natural fats are described in this paper.

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1) Presented at the annual meeting of the Japan Oil Chemists' Society, Nagoya, November 8, 1955.

2) Y. Inouye and M. Noda, *J. Agr. Chem. Soc. Japan*, **27**, 50 (1953).

3) H. K. Mangold, B. G. Lamp, and H. Schlenk, *J. Am. Chem. Soc.*, **77**, 6070 (1955).

4) V. Kobrle and R. Zahradník, *Chem. Listy*, **48**, 1703 (1954).

5) Y. Inouye and M. Noda, This Bulletin, **19**, 214 (1955).

6) Y. Inouye, O. Hirayama, and M. Noda, *J. Japan Oil Chemists' Soc.*, **5**, 16 (1956).

7) Y. Inouye, M. Noda, and O. Hirayama, *J. Am. Oil Chemists' Soc.*, **32**, 132 (1955).

EXPERIMENTAL

Fatty Acids. The pure fatty acids were prepared by the same procedure as reported in the previous paper.[8] 9-Decenoic, 9-dodecenoic, 9-hexadecenoic, and behenic acids were employed in the forms of their highly concentrated fractions obtained from natural sources after fractional distillation.

Preparation of *p*-Bromophenacyl Ester 2, 4-Dinitrophenylhydrazones. The derivatives were obtained in pure states for the determinations of their R_F values by the method described in the previous paper. However, a modified method of the preparation was employed for mixed fatty acids.

A solution of mixed fatty acids (about 20 mg) in 1 ml of alcohol was neutralized with a 0.05 N potassium hydroxide solution in alcohol in the presence of phenolphthalein, and then slightly acidified with one drop of 0.02 N methanolic hydrochloric acid. To the solution was added a slightly smaller amount of *p*-bromophenacyl bromide than that calculated from the above titration volumes. After

8) Y. Inouye, O. Hirayama, and M. Noda, This Bulletin, **20**, 5 (1956).

TABLE I

R_F VALUES OF *p*-BROMOPHENACYL ESTER 2, 4-DINITROPHENYLHYDRAZONES OF FATTY ACIDS[a]

Acid	Solvent System[b]							
	MAP		EAD		BEAD		MSAO	
	N[c]	M[d]	N[c]	M[d]	N[c]	M[d]	N[c]	M[d]
Acetic	0.71	...	0.81	...	0.91	...	0.67	...
Butyric	0.69	...	0.71	...	0.80	...	0.61	...
Caproic	0.63	...	0.62	...	0.71	...	0.55	...
Caprylic	0.55	...	0.53	...	0.61	...	0.49	...
Capric	0.48	...	0.44	...	0.51	...	0.42	...
Lauric	0.41	...	0.35	...	0.41	...	0.35	...
Myristic	0.35	...	0.27	...	0.32	...	0.29	...
Palmitic	0.29	...	0.19	...	0.23	...	0.23	...
Stearic	0.23	...	0.13	...	0.16	...	0.17	...
Arachidic	0.17	...	0.07	...	0.10	...	0.13	...
Behenic[e]	0.12	...	0.05	...	0.07	...	0.09	...
9-Decenoic[e]	0.53	0.83	...	0.83	...	0.93	...	...
10-Undecenoic	0.50	0.77	0.47	0.78	0.51	0.86	0.44	0.52
9-Dodecenoic[e]	0.48	0.73	...	0.73	...	0.81	...	...
9-Tetradecenoic[e]	0.41	0.68	...	0.60	...	0.68	...	...
9-Hexadecenoic[e]	0.34	0.57	...	0.46	...	0.55	...	...
Oleic	0.28	0.51	0.18	0.38	0.20	0.42	0.24	0.29
Erucic	0.17	0.39	0.09	0.23	0.09	0.19	0.16	0.19
Linoleic	0.33	0.72	0.23	0.61	0.22	0.66	0.27	0.40
Linnolenic	0.38	0.69	0.29	...	0.26	...	0.29	0.48
Elaidic	0.26	0.52	0.17	0.42	0.17	0.43	0.24	0.31
Brassidic	0.16	0.45	0.07	0.27	0.10	0.25	0.15	0.21
Stearolic	0.32	0.46	0.27	0.50	0.22	0.45	0.28	0.38
Behenolic	0.22	0.35	0.14	0.31	0.11	0.30	0.23	0.26
Ricinoleic	0.70	0.77	0.67	0.71	0.74	0.79	0.50	0.57

a Ascending chromatography at 30°.
b Solvent system: MAP, methanol–acetic acid–petroleum hydrocarbon; EAD, ethanol–acetic acid–decalin; BEAD, buthanol–ethanol–acetic acid–decalin; MSAO, methanol–ethyl acetate–acetic acid–olive oil.
c Non-mercurated derivatives.
d Mercurated derivatives.
e Contaminated with other acids having no effect on the R_F values.

refluxing for one hour followed by cooling to 5—30°, the reaction mixture was added dropwise to a slight excess of 0.5% solution of 2, 4-dinitrophenylhydrazine in 2 N methanolic hydrochloric acid, with stirring, and allowed to stand for from two to three hours at 10°. Four ml of ether and then 10 ml of water were added to the mixture. The ethereal layer was used as a sample of the chromatographic separation when the original fatty acids consisted of only saturated acids. In the case of a mixture of both saturated and unsaturated acids, the ether extract was further washed free of hydrochloric acid, dried over anhydrous sodium sulphate, and divided into two equal portions. The first sample solution was prepared from one portion by adding 1 ml of benzene and 10 ml of water, and the second sample solution from the other by mercurating as described below. These two samples were employed in combination for the present method.

Mercuration of *p*-Bromophenacyl Ester 2, 4-Dinitrophenylhydrazones of Unsaturated Acids. To about 1 ml of the dried, ethereal solution of the derivatives obtained above was added a 50—100% excess of mercuric acetate than that calculated from the iodine numbers of the original acids and 2 ml of absolute methanol. After refluxing for thirty minutes at 60 , 1 ml of benzene and then 10 ml of water were added to the reaction mixture. The upper benzene layer was submitted to chromatographic separation.

Paper Chromatography. *Solvent systems.* Petroleum hydrocarbon (b. p. 140—170°), decalin, and olive oil were used for the stationary phases. As the moving solvent, methanol—acetic acid—petroleum hydrocarbon (5:1:1 by volume) (MAP) was used against petroleum hydrocarbon, 90% ($^{v}/_{v}$) aqueous ethanol—acetic acid—decalin (10:2:1) (EAD) or *n*-butanol—80% aqueous ethanol—acetic acid—decalin (2:8:2:1) (BEAD) against decalin, and 90% aqueous methanol—ethyl acetate—acetic acid (10:5:1) (MSAO) against olive oil.

Procedure. Sample solutions from 0.5 to 5 μl, containing 2—50 μg of each derivative of the fatty acids, were placed on a large sheet of Tōyō No. 2 filter paper. The paper was uniformly sprayed with the stationary solvent, petroleum hydrocarbon or decalin, the amount being 14 ml per 1000 cm^2 of the paper with the former solvent, and 5.7 ml with the latter. In the case of solvent system MSAO, the stationary solvent was applied by dipping the paper into a 5% solution of olive oil in benzene, and the paper was spotted with samples after evaporating the benzene at room temperature. The chromatogram was developed with the moving solvent by the ascending technique at 30°. The development time required for a satisfactory separation was 5 hours in system MAP or MSAO, and 7 hours in EAD or BEAD. All the derivatives of saturated and unsaturated acids were detected as yellow spots on the chromatogram. By spraying with a 0.2% solution of diphenylcarbazone in alcohol, the spots of unsaturated acid derivatives were selectively changed into green color. The R_F values of the ester derivatives and their mercuric compounds are given in Table I, and the chromatograms are shown in Figures 1 and 2.

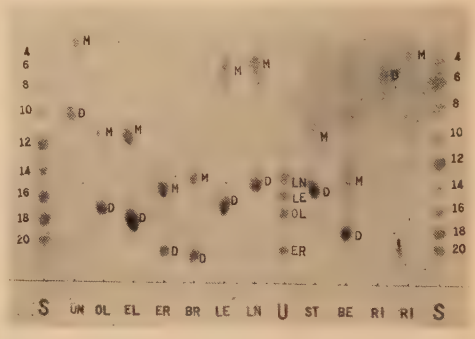

FIG. 1. Chromatogram of *p*-Bromophenacyl Ester 2, 4-Dinitrophenylhydrazones of Fatty Acids.

Solvent system: MAP, The spot identifications are as follows: number, the number of carbon atoms in the acid; D, the ester derivatives; M, mercuric compounds of the ester derivatives; UN, undecenoic; OL, oleic; EL, elaidic; ER, erucic; BR, brassidic; LE, linoleic; LN, linolenic; ST, stearolic; BE, behenolic; RI, ricinoleic. S indicates a mixture of saturated acid derivatives; and U, a mixture of unsaturated acid derivatives.

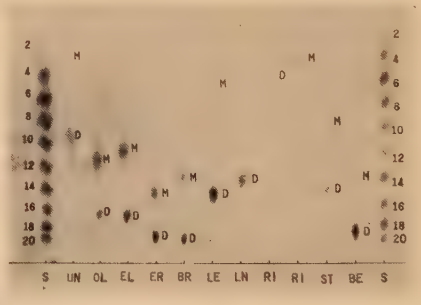

FIG. 2. Chromatogram of *p*-Bromophenacyl Ester 2, 4-Dinitrophenylhydrazones of Fatty Acids.

Solvent system: EAD. The spot identifications are the same as those in Fig. 1.

Analysis of Natural Mixed Fatty Acids. The analysis of the component fatty acids of natural fats

were attempted by the paper-chromatographic separation using system MAP and EAD.

The mixed fatty acids obtained from olive oil were fractionated by the distillation procedure. Each fraction thus obtained was derived into the two sample solutions as described above, respectively. Approximately equivalent amounts of the two samples were developed in parallel with the standard acid derivatives on the same paper. The component acids were identified by comparison of R_F values with the standard spots, coupling the coloration by diphenylcarbazone and observation of the differences in amount between the corresponding spots of the two samples. On the other hand, two samples directly prepared from the original fatty acid mixture of the olive oil were also subjected to the chromatographic separation in the same way. Comparison between the former and the latter results is shown in Table II.

TABLE II

SEPARATION AND IDENTIFICATION OF THE COMPONENT FATTY ACIDS OF NATURAL FATS BY PAPER CHROMATOGRAPHY

Fat[a]	Olive oil				Coconut oil	Rape oil		Soybean oil		Cow milk fat
	A[b]		B[c]		B[c]	B[c]		B[c]		B[c]
Acid	R_F values of spots detected[d]									
Butyric	...	...	...	...	...	...	...	...	...	0.70
Caproic	...	...	...	...	0.64^{VW}	...	...	...	...	0.64^{VW}
Caprylic	...	...	...	...	0.56^{S}	...	...	...	...	0.57^{VW}
Capric	...	...	...	...	0.47^{S}	...	...	...	...	0.50^{VW}
Lauric	...	...	...	...	0.41^{VS}	...	...	...	...	0.43
Myristic	0.35^{VW}	...	0.36^{VW}	...	0.34^{S}	0.34^{VW}	...	0.35^{VW}	...	0.35^{S}
Palmitic	0.29^{S}	...	0.29^{S}	...	0.28	0.27	...	0.27^{S}	...	0.28^{S}
Stearic	0.21	...	0.23	...	0.23^{VW}	0.22^{VW}	...	0.22	...	0.22
Arachidic	0.16^{VW}	...	...	...	...	0.16^{VW}	...	0.17^{VW}	...	...
Behenic	...	...	...	...	...	0.11^{VW}	...	0.11^{VW}	...	...
9-Hexadecenoic	0.34	0.58^{VW}	...	...	...	...	...	...	...	...
Oleic	0.28	0.51^{VS}	0.26	0.49^{VS}	0.51	0.28	0.51^{S}	0.27	0.50^{S}	0.50^{VS}
Eicocenoic	...	...	...	...	...	0.22	0.45	...	...	...
Erucic	...	...	...	...	...	0.17	0.38^{VS}	...	...	...
Linoleic	0.32	0.67	0.33	0.68	...	0.33	0.67	0.33	0.69^{VS}	...
Linolenic	...	...	...	...	...	0.38	...	0.38	...	...

a Olive oil: A. V., 4.1; Sap. V., 195.7; I. V., 81.0, Coconut oil: A. V., 3.7; Sap. V., 258.9; I. V., 8.5. Rape oil: A. V., 2.7; Sap. V., 180.4; I. V., 94.7. Soybean oil: A. V., 2.1; Sap. V., 194.8; I. V., 133.7. Cow milk fat: A. V., 0.5; Sap. V., 225.2; I. V., 39.5.

b Analyzed through fractionation by the distillation procedure.

c The original fatty acid mixture was analyzed without the fractionation step.

d Ascending chromatography at 30°. Solvent system: MAP. Classification of compounds: those underlined, mercuric acetate addition compounds; others, non-mercuric compounds. Color intensity of spots: vs, very strong; s, strong; vw, very weak.

Coconut oil, rape oil, soybean oil, and cow milk fat were analyzed by the latter procedure without the fractionation step. The results are given in Table II and Figures 3 and 4.

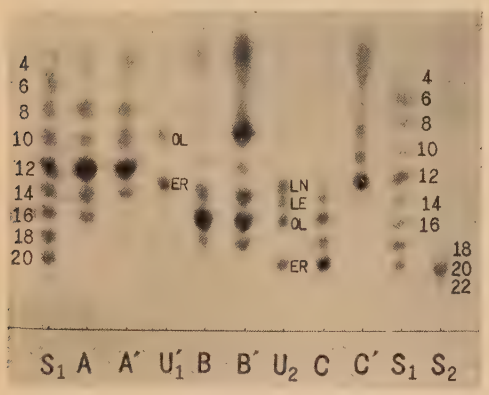

FIG. 3. Chromatogram of *p*-Bromophenacyl Ester 2, 4-Dinitrophenylhydrazone of Natural Fatty Acid Mixtures.

Solvent system: MAP. S_1 and S_2 indicate a mixture of pure saturated acid derivatives; and U_1 and U_2, a mixture of pure unsaturated derivatives. A, coconut oil; B, cow milk fat; C, rape oil. The symbol (') designates the sample treated with mercuric acetate. The identifications of standard spots are as follows; number, the number of carbon atoms in the acid; OL, oleic; ER, erucic; LE, linoleic; LN, linolenic.

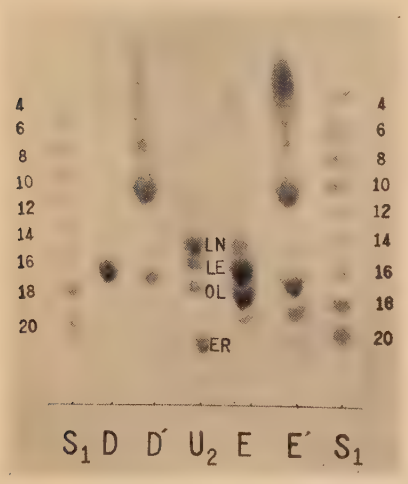

FIG. 4. Chromatogram of *p*-Bromophenacyl Ester 2, 4-Dinitrophenylhydrazones of Natural Fatty Acid Mixtures.

Solvent system: MAP. D, olive oil; E, soybean oil. The treatments of samples and the identifications of standard spots are the same as those in Fig. 3.

RESULTS AND DISCUSSION

The hydrazone derivatives might be expected to contain *syn-* and *anti*-isomers, as reported in the previous paper.[8] However, the benzene extract of all hydrazone products derived from pure acid shows only one spot on the chromatogram according to present technique. This fact proves that the derivatives of fatty acids are applicable for the chromatographic separation of natural fatty acid mixture, with additional advantage of their easy preparation in almost theoretical yields.

As seen in Table I, the R_F values increase with shorter chain length, higher unsaturation, and more hydroxylation of fatty acids in the derivatives. With system MAP it is shown that, in relation to a saturated compound, the increment for one double bond is approximately equal to that found for shortening the chain length by one ethylene group; and the increment for one triple bond, two ethylene groups. Similar relations of shifts are also observed with the other systems. Higher R_F values of the mercuric compounds of unsaturated derivatives are found on the chromatogram of system MAP, where monoethenoid acids turn up in the region of the saturated acids containing 8 less C-atoms than the corresponding acids, and both linoleic and linoenic acids in the region of butyric acid. In systems MAP, EAD, and BEAD, a considerable difference in shifts between *cis-* and *trans*-isomers was displayed with the hydrazone derivatives and their mercuric compounds (Figures 1 and 2).

Among the solvent systems used, MAP is most suitable for general separations of the mixtures containing saturated and unsaturated acids. It shows good separations of even saturated acids from C_4 to C_{22}, even monoethenoid acids from C_{10} to C_{22}, and C_{18} series from stearic to linolenic in the derivatives, as well as even monoethenoid acids from C_{10} to C_{22} in the mercuric compounds, and

that the latter unsaturated compounds have high R_F values sufficient to separate themselves from the former saturated derivatives. All the other systems are also effective for separations of even saturated acids from C_2 to C_{20} and even monoethenoid from C_{10} to C_{22}, but EAD and BEAD have a disadvantage for identification of linoleic and linolenic acids. The spots of the mercuric compounds of diethenoid, triethenoid, and monoethenoid acids are diffused on use of system MAP, particularly EAD and BEAD. The best separation of these compounds was sometimes displayed with system MSAO, but it did not give reproducible results.

The practical applications of the present technique have given satisfactory results. All the component acids reported in the literature were successfully identified in each case of olive oil, coconut oil, rape oil, and soybean oil. These results show that fractionation process in the preparation of starting material is not required in general analysis of such oils with relatively simple composition. In the case of cow milk fat with more complicated composition, the identification of the component acids was also well performed with a small quantity of the original acid mixture, excluding the minor component of unsaturated acids.

From these results, it appears that the method using system MAP is valid for the identification of the component acids more than about 0.2% of the total acids, but not effective for the unsaturated acids of an amount less than about 4% of the total acids only when lower saturated acids interfere with these unsaturated acids. For the analysis of lower fatty acids, system EAD is more effective than system MAP, because the detection of fatty acids lower than caproic acid are often suffered in the latter system, by a tailing spot which may be attributed to an excess of 2,4-dinitrophenylhydrazine and small decomposed products.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 206~210, 1956]

Isolation and Identification of Taraxasterol and β-Amyrin from the Bird-lime of *Balanophora japonica* Makino*

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Received June 25, 1956

In the present work, two triterpene alcohols, taraxasterol, $C_{30}H_{50}O$, m.p. 220-221°, $[\alpha]_D^{12}$+93.5° (acetate, m.p. 248–249°; benzoate, m.p. 242°), and β-amyrin, $C_{30}H_{50}O$, m.p. 196°, $[\alpha]_D^{13}$+86.2° (acetate, m.p. 239–240°; benzoate, m.p. 232°) were lsolated from the unsaponifiable fraction of the ethereal extract of *Balanophora japonica* Makino (*Balanophoraceae*). Taraxasterol was isomerized to yield ψ-taraxasterol (heterolupeol), $C_{30}H_{50}O$, m.p. 208–209°, which was further characterized as its acetate, m.p. 238–240°.

Palmitic acid was also isolated from the fatty acid-fraction.

Balanophora japonica Makino (Japanese name: Tsuchi-tori-mochi; *Balanophoraceae*) is a plant indigenous to the Southern part of Japan, and Loochoo, being well-known to taxonomists because of its curious ecological behaviour. It was reported[1] that this plant is parasitic to the terminal roots of parent plants, such as *Bobua myrtacea* Siebold et Zuccarini (Japanese name: Hai-no-ki; *Symplocaceae*) and *B. japonica* Miers (Japanese name: Kuroki). Besides, it has recently been reported by Y. Shimada*** that this plant is also found to be capable of being parasitic to the terminal roots of *Distylium racemosum* Siebold et Zuccarini (Japanese name: Isu-no-ki; *Hamamelidaceae*).

In 1911, the isolation from *Balanophora elongata* Blume of a compound, balanophorin (m.p. 55–57°) was described by M. Simon[2], and it was reported to yield palmitic acid on destructive distillation under diminished pressure. In 1926, balanophorin (m.p. 77°) was further isolated in pure form by A.J. Ultée[3] from the same plant, and was proved by degradation and synthesis to be β-amyrin palmitate.

To date, to our knowledge, nothing has yet been reported as having been isolated from *Balanophora japonica* Makino.

The present paper deals with the results of experiments which have been carried out for the isolation and identification of the triterpenoid constituents of this plant.

To achieve complete separation of the triterpenoids, the following procedure of fractionation has been shown to be expedient.

The unsaponifiable fraction of the ethereal extract obtained from this plant was acetylated to yield a mixture of taraxasteryl acetate and β-amyrin acetate, which was further fractionally recrystallized successively from alcohol and ethyl acetate. From the fraction sparingly soluble in alcohol, taraxasteryl acetate[4] was

* This is Part III of a series o papers entitled as "Studies of Constituents of Various Sorts of Bird-lime"; Presented at the Extraodinary General Meeting of the Agricultural Chemical Society of Japan held at Hiroshima, Nov. 3, 1955; Promptly communicated in this Bulletin. **20**, 97 (1956). Part II: *J. Pharm. Soc. Japan*, **74**, 422 (1954).

** T. Makino, *Bot. Mag. Tokyo*, **23**, 23 (1909).

1) K. Watanabe, *J. Japanese Bot.*, **18**, 250, 293, 382, 438 (1942).

*** Y. Shimada of Kemmotsudai Arboretum of Kumamoto Forestry Bureau, Kumamoto, Japan: Private communication to the author.

2) in M. Simon, *Monatsh.*, **32**, 89 (1911).

3) A.J. Ultée, *Bull. Jardin. Bot. Buitenzorg*, (3), **8**, No. 1, 3 (1926); *Chem. Zentr.*, **1927**, II 95; cf. T. Poleck, *Ann.*, **67**, 179 (1948).

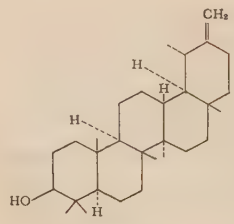
4) According to the private communication to the author from Prof. Dr. E.R.H. Jones of University of Manchester, England, taraxasteryl acetate and benzoate isolated by the author in this investigation have definitely been confirmed to be identical with the authentic samples of taraxasteryl acetate of Dr. E.R.H. Jones, and taraxasteryl benzoate of the late Dr. J.C.E. Simpson, by mixed melting point determinations as well as by comparison of the infra-red spectra of taraxasteryl acetates.

The authors is now deeply indebted to Prof. Dr. E.R.H. Jones for his courtesy of determining the mixed melting points and measuring the infra-red spectra.

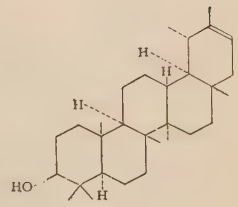
isolated in pure form, $C_{32}H_{52}O_2$, m.p. 248–249°, $[\alpha]_D^{12}+100.0°$, deacetylation of which afforded taraxasterol, $C_{30}H_{50}O$, m.p. 220–221°, $[\alpha]_D^{12}+93.5°$ (benzoate[4], $C_{37}H_{54}O_2$, m.p. 242°, $[\alpha]_D^{12}+106.5°$).

It was further identified as taraxasterol by conversion into ψ-taraxasterol (heterolupeol), $C_{30}H_{50}O$, m.p. 208–209°, $[\alpha]_D^{22}+48.5°$ (acetate, $C_{32}H_{52}O_2$, m.p. 238–240°, $[\alpha]_D^{12}+56.2°$).

As shown in the following figure, the infrared spectra of taraxasterol and its acetate exhibited a band at 876 cm^{-1} and at 882 cm^{-1}, respectively, indicating the presence of the group $\begin{matrix}R_1\\R_2\end{matrix}\!\!>\!C=CH_2$, whereas those of ψ-taraxasterol and its acetate exhibited a band at 777 cm^{-1} and at 782 cm^{-1}, respectively[5], indicating the presence of the group $\begin{matrix}R_1\\R_2\end{matrix}\!\!>\!C=C\!<\!\begin{matrix}H\\R_3\end{matrix}$, and the absence of the group

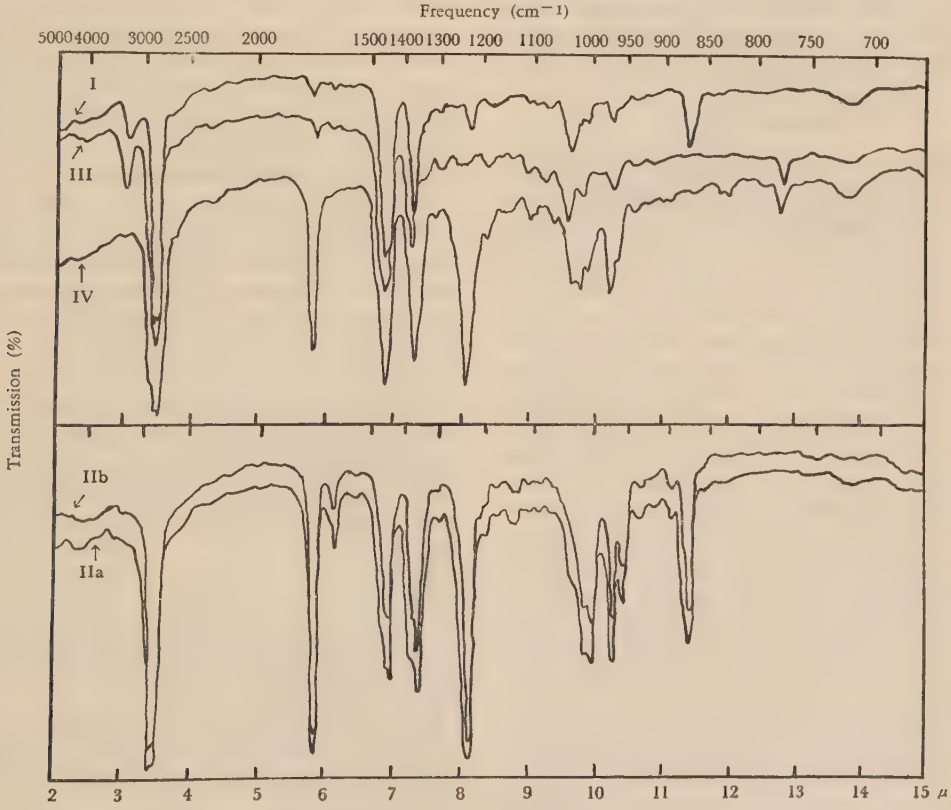


Taraxasterol



ψ-Taraxasterol (Heterolupeol)

5) T.R. Ames, J.L. Beton, A. Bowers, T.G. Halsall, E.R.H. Jones, *J. Chem. Soc.*, **1954**, 1905. They report 778 cm^{-1} and 780cm^{-1}, respectively.



(I): Taraxasterol; (IIa): Taraxasteryl Acetate; (IIb): Taraxasteryl Acetate of Prof. Dr. E.R.H. Jones; (III): ψ-Taraxasterol (Heterolupeol); (IV): ψ-Taraxasteryl Acetate.

$R_1 \diagdown C=CH_2$.
$R_2 \diagup$

From the fraction freed of taraxasteryl acetate, there was isolated β-amyrin acetate, $C_{32}H_{52}O_2$, m.p. 239°, $[\alpha]_D^{18}+83.3°$, deacetylation of which afforded β-amyrin, $C_{30}H_{50}O$, m.p. 196°, $[\alpha]_D^{13}+86.2°$ (benzoate, $C_{37}H_{54}O_2$, m.p. 232°).

To date, taraxasterol has been reported to be present in several plants of the family *Compositae*, such as *Taraxacum officinale* Linnaeus[6], *Andryala canariensis*[7], *Lactuca virosa* Linnaeus[8], *Cynara Scolymus* Linnaeus[9], *Anthemis nobilis* Linnaeus[6,10], etc.[11], and in *Euphorbia tirucalli* Linnaeus[12] (*Euphorbiaceae*).

It is of interest to note the fact that the presence of taraxasterol along with β-amyrin has now been proved for such a rare plant as *Balanophora japonica* Makino.

EXPERIMENTAL*

Isolation and Hydrolysis of the Resin (Birdlime). The rhizomes and flowers (1.56 kg) of *Balanophora japonica* Makino, collected in Kagoshima Prefecture in the winter of 1954, were cut into chops, air-dried, and thoroughly dried at 60° for 10 hrs. Yield, 435.0 g (28.24%).

After exhaustive continuous extraction of the dried material with ether for 60 hrs., there was obtained 71.0 g (16.32% of the dried matter) of the orange-colored extract soluble in ten times its weight of cold benzene. (acid value, 2.5; sap. value, 13.7; iodine value, 55.0).

After being refluxed with 15% benzene-alcoholic potassium hydroxide solution (alcohol-benzene: 1 : 1) for 60 hrs., the mixture was filtered while hot. After removal of the solvent *in vacuo*, the residue was diluted with water (2.5 l), and subjected to continuous extraction with ether for 30 hrs. The aqueous layer was combined with the washings, acidified with hydrochloric acid, then extracted with ether to yield a fatty acid-fraction (21.5 g: 4.94% of the dried matter) (I), whereby no separation of the potassium salt of triterpene acid was observed between alkaline and ethereal layers.

After drying and removal of ether, there remained a pale yellow crystalline mass (49.0 g: 11.26% of the dried matter), which was refluxed with 25 times its weight of alcohol for 1 hr., cooled and filtered. The filtrate was concentrated to a small volume, and refluxed with an appropriate amount of alcohol, as above.

After repeating the procedure, there were finally obtained (a) a yellowish syrupy fraction (II), (which is easily soluble in alcohol and becomes semisolid after a week), (27.6 g: 6.34% of the dried matter), and (b) colorless needles, m.p. 164–166° (21.3 g: 4.89% of the dried matter) (III).

Isolation of Taraxasteryl Acetate. To a solution of (III) in 50 ml of benzene, was added 20 ml of acetic anhydride, and the mixture was refluxed for 2 hrs. After removal of the solvents *in vacuo*, the crude acetate was washed with water, dried, and refluxed with 45–50 times its weight of alcohol to yield a clear solution, indicative of the absence of triterpenoids, such as taraxol[6b] or ilexol[13].

After removal of alcohol, the residue was recrystallized from ethyl acetate to yield scales (4.5 g), m.p. 230–235°, which after further recrystallization afforded glistening scales (3.7 g), melting at 248–249° either alone or on admixture with an authentic specimen[4]. $[\alpha]_D^{12}+100.0°$ (c, 0.635).

Anal. Found: C, 81.55; H, 10.10. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; 11.18.

Taraxasterol. One gram of taraxasteryl acetate was refluxed with 5% alcoholic potassium hydroxide solution for 2 hrs.

After removal of alcohol, the residue was diluted with water, and extracted with ether. The ethereal layer was washed with water, dried and distilled.

6) a) F.B. Power, H. Browning, Jr., *J. Chem. Soc.*, **101**, 2411 (1912); b) S. Burrows, J.C.E. Simpson, *ibid.*, **1938**, 2402; c) J. Zimmermann, *Helv. Chim. Acta*, **28**, 127 (1945).

7) M. Vilkas, *Compt. rend.*, **235**, 179 (1952).

8) O. Hesse, *Ann.*, **234**, 243 (1886); G. Hesse, E. Eilbracht, F. Reicheneder, *ibid.*, **546**, 233 (1941); J.C.E. Simpson, *J. Chem. Soc.*, **1944**, 283.

9) W. Fruhstorfer, *Chem. Ber.*, **87**, 423 (1954).

10) T. Klobb, *Bull. soc. chim.*, (3), **27**, 1229 (1902); *Compt. rend.*, **148**, 1272 (1902); *Ann. chim. phys.*, (8), **18**, 135 (1909); cf. N.H. Cohen. *Arch. Pharm.*, **246**, 520 (1908).

11) J. Zellner, *Monatsh.*, **47**, 681 (1927); J. Grzgybowska et al., *Chem. Abstr.*, **48**, 12378 (1954).

12) D.W. Daines, F.L. Warren, *J. Chem. Soc.*, **1949**, 2554.

* Melting points were corrected; Rotations were measured in $CHCl_3$ solution, using 1-dm tubes.

13) A new triterpene alcohol first isolated by S. Iseda (*J. Pharm. Soc. Japan*, **72**, 1064 (1951)) from the commercial "white birdlime, the source of which is said to be of the bark of *Ilex integra* Thunberg (Japanese name: Mochi-no-ki; *Aquifoliaceae*).

After recrystallizations thrice from alcohol of the residue, there were obtained colorless long soft needles (0.8 g), melting at 220–221°, $[\alpha]_D^{12}$+93.5° (c, 0.852).

Anal. Found: C, 84.04; H, 11.82. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

Taraxasteryl Benzoate. To a solution of 0.1 g of taraxasterol (m.p. 220–221°) in 1 ml of pyridine, was added 1 ml of benzoyl chloride drop by drop under cooling, and the mixture was refluxed for 30 min. After being left overnight, the mixture was poured onto an ice-cooled solution of dilute sulfuric acid, and the crude benzoate separated was extracted with ether. The ethereal solution was washed successively with dilute sodium carbonate solution and water, and dried. After removal of ether, the residue was recrystallized thrice from acetone-benzene (1 : 1), giving flat needles (0.5 g), which melted at 242° either alone or on admixture with an authentic specimen[4]. $[\alpha]_D^{12}$+106.5° (c, 0.595).

Anal. Found: C, 83.11; H, 10.28. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H, 10.25.

This benzoate (0.3 g) was refluxed with 5% alcoholic potassium hydroxide solution for 2 hrs. to yield free alcohol (0.25 g), which after recrystallizations thrice from alcohol, melted at 220–221° either alone or on admixture with taraxasterol obtained as above.

Isomerisation of Taraxasterol into ψ-Taraxasterol (Heterolupeol). To a solution of 1.0g of taraxasteryl acetate (m.p. 248–249°) in 25 ml of benzene, was added a mixture of 5 ml of sulfuric acid (d. 1.84) and 50 ml of alcohol, and the resulting solution was refluxed for 5 hrs. After dilution with cold water, the reaction mixture was extracted with ether, and the ethereal solution was thoroughly washed with water, and dried. After removal of ether, the residue was recrystallized thrice from ethyl acetate-alcohol (1 : 1) to yield soft needles (0.8 g), m.p. 208–209°, $[\alpha]_D^{12}$+48.5° (c, 0.652).

Anal. Found: C, 84.47; H, 11.73. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

ψ-Taraxasteryl Acetate. ψ-Taraxasterol (0.5 g; m.p. 208–209°) was acetylated with 2 ml of acetic anhydride and a few drops of pyridine. After being diluted with water, the crude acetate separated was extracted with ether. After recrystallizations several times from ethyl acetate-alcohol (1 : 1) of the crude acetate, there was obtained the pure acetate which crystallized in leaflets (0.45 g) and melted at 238–240°, $[\alpha]_D^{12}$+56.2° (c, 0.752).

Anal. Found: C, 81.81; H, 11.12. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

This acetate (0.2 g) was refluxed with 5% alcoholic potassium hydroxide solution to yield free alcohol (0.18 g), which after recrystallizations thrice from ethyl acetate-alcohol (1 : 1), melted at 209°, either alone or on admixture with ψ-taraxasterol obtained as above.

Isolation of β-Amyrin Acetate. a) The residue freed of taraxasteryl acetate (16.8 g) was recrystallized several times from alcohol or from ethyl acetate to yield long prismatic crystals (0.65 g), m.p. 239–240°. b) To a solution of 27.6 g of the yellowish syrupy fraction (II) in 50 ml of benzene, was added 25 ml of acetic anhydride, and the mixture was refluxed for 2 hrs. After removal of the solvents *in vacuo*, the crude acetate was dissolved in 100 ml of glacial acetic acid, and left in the refrigerator for several days. There separated granular crystals, which were filtered, washed with water, dried, and recrystallized several times from alcohol to give long prismatic crystals (5.5 g), m.p. 239°. Both of the samples melted at 239–240° on admixture with an authentic specimen of β-amyrin acetate[14]. $[\alpha]_D^{13}$+83.3° (c, 0.665).

Anal. Found: C, 82.02; H, 11.32. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

β-Amyrin. Two grams of β-amyrin acetate (m.p. 239–240°) was refluxed with 5% alcoholic potassium hydroxide solution, and the resulting solution was diluted with a sufficient amount of water, and thoroughly extracted with ether. The ethereal solution was washed with water, and dried. After removal of ether, the residue was recrystallized thrice from alcohol, giving colorless long prisms (1.8 g), m.p. 196°. $[\alpha]_D^{15}$+86.2° (c, 0.583).

Anal. Found: C, 84.22; H, 11.95. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

β-Amyrin Benzoate. A solution of 0.8 g of β-amyrin (m.p. 196°) in 1 ml of pyridine was benzoylated as usual with 1.5 ml of benzoyl chloride. After several recrystallizations from ethyl acetate and then from benzene-acetone (1 : 1) of the crude benzoate, there were obtained colorless long prisms melting at 232°. Yield, 0.7 g.

Anal. Found: C, 83.51; H, 10.60. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H, 10.25.

Hydrolysis of 0.3 g of this benzoate with 5% alcoholic potassium hydroxide solution afforded free alcohol (0.28 g), which recrystallizations thrice from alcohol, melted at 195–196° either alone or on admixture with an authentic specimen of β-amyrin[14].

14) S. Iseda, K. Yagishita, N. Toya, *J. Pharm. Soc. Japan*, **74**, 422 (1954).

Palmitic Acid. An amount of 21.5 g of the crude fatty acid fraction (I), was dissolved in a small volume of alcohol and left in the refrigerator for several days. There separated granular crystals, which after several recrystallizations from alcohol, afforded glistening scales and melted at 61–62°. Yield, 5.5 g.

Anal. Found: C, 74.53; H, 12.62. Calcd. for $C_{16}H_{32}O_2$: C, 74.94; H, 12.58.

The amide of this acid (2 g) was prepared as usual and recrystallized several times from alcohol to yield lamellar crystals (2.0 g), which melted at 106° either alone or on admixture with an authentic specimen of palmitic acid amide.

Anal. Found: C, 75.39; H, 13.04; N, 5.50. Calcd. for $C_{16}H_{33}ON$: C, 75.23; H, 13.02; N, 5.48.

The author's heartiest thanks are due to Prof. S. Iseda of this Laboratory, to Prof. Dr. Y. Oshima of Kyushu University, and to Prof. Dr. E. Sebe of Kumamoto Universtity for their helpful advice and encouragement given throughout the whole course of this work. He is also deeply indebted to Messrs. H. Kozuma and K. Kawasaki of the Technical Department, Minamata Factory, Shin Nippon Chisso Hiryo. Co., Minamata, Kumamoto, for their courtesy of measuring infra-red spectra, to Mr. Y. Shimada of Kemmotsudai Arboretum of Kumamoto Forestry Bureau, Kumamoto, for determining the proper classification of the plant, to Mr. Y. Mayeda of Yunozuru Middle School, Minamata, Kumamoto, and to Miss. E. Nakayama of Affiliated Middle School of Kagoshima University, for collecting the plant material used in the present work.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 211~214, 1956]

Phytopathological Chemistry of Black Rot Sweet Potato

Part XXVI. Activity of Proteolytic Enzymes in the Diseased Sweet Potato

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The proteolytic enzyme system in the tissues of sweet potato root was partially purified and its several characteristics were investigated. Based on the results from these preliminary experiments, we found that proteolytic activity in the tissues suffering from black rot declines as penetration of the pathogen proceeds. Procedures to extract the enzyme system from the affected plant tissues were also established. The diminution in activity of the affected tissues was discussed in connection with those results reported hitherto.

It has previously been shown that an increase in the respiratory-rate is observed in the healthy part next to the affected site when sweet potato is infected with *Ceratostomella fimbriata*.[1] This respiratory increase is accompanied with the reduction of the inorganic phosphate level and concomitant increase of organic phosphates in that same part.[2] Together with the results obtained from the experiment in which the effects of 2,4-dinitrophenol and other uncoupling substances have been tested on the respiratory increase,[3] we have postulated that a considerable portion of this respiratory increase may be closely connected with the oxidative phosphorylation. In line with these experiments, the ratio of acid insoluble to acid soluble-nitrogen in the part mentioned above has been investigated resulting that the ratio gradually increases as infection proceeds, while the total nitrogen level of the mentioned part remains constant.

These results have led us to the conclusion that sweet potato tissues may respond to disease development with new protein synthesis and perhaps accompany rearrangement of the reserved proteins. By all mean, these active processes call for energy supply from, for example, ATP, and this requisition, in turn, stimulates the increase of the ADP level, which is a limiting principle of the respiratory-rate.[3,4,5,6] This causes an increase in the oxygen uptake in the long run. Some evidences are at present available which seem to support this picture; in black rot sweet potato, oxidizing enzymes such as polyphenol oxidase, cytochrome oxidase and peroxidase are activated to a considerable degree.[7] Furthermore, it has recently been found that in the sound part adjacent to the infected, protein fractions electrophoretically differ from those prepared from the healthy tissues, and the nitrogen amount of the cytoplasmic particulates, such as mitochondria increases.[8] Thus, functional proteins either synthesized or reorganized, appear to be of particular importance to comprehend the biochemical mechanism of the host-parasite interaction.

In the present investigation, the alteration of

1) I. Uritani and S. Takita, *J. Agr. Chem. Soc. Japan*, **27**, 168 (1953).

2) T. Akazawa and I. Uritani, *ibid*, **29**, 381 (1955).
T. Akazawa and I. Uritani, *Nature*, **176**, 1071 (1955).

3) I. Uritani, T. Akazawa and M. Uritani, *ibid*, **124**, 1060 (1954).

4) F. Lynen, *Ann.*, **546**, 120 (1941).

5) M.J. Johnson, *Science*, **94**, 200 (1941).

6) P. Siekevitz and V. Potter, *J. Biol. Chem.*, **201**, 1 (1953).

7) I. Uritani and T. Akazawa, *J. Agr. Chem. Soc. Japan*, **27**, 789 (1953).

8) T. Akazawa, *J. Biochem.*, in press.

activity of proteolytic enzymes which may take part in the protein metabolism of black rot sweet potato has therefore been studied. Investigations concerning the endoproteases of higher plants have scarcely been done, except for a few plants. Studies dealing with the change of proteolytic activities in higher plants in terms of disease resistance are also meager. Preliminary experiments have, therefore, been undertaken to examine several characteristics of this enzyme system so that they will serve for our end to investigate the changes of proteolytic activity in the sweet potato tissues suffering from black rot.

METHODS

Fresh sweet potato tissues (Norin No. 2 var.) were pealed, chopped and homogenized with a three fold volume of 0.04 M phosphate buffer, centrifuged at 170×g, 10 min. For the estimation of the effect of pH upon proteolytic activity, a supernatant solution as follows was employed. A couple of reaction mixtures containing enzyme solution, buffer of a definite pH and 5% casein, in equal volume were prepared. One of them was deproteinized with an equal volume of 0.6 M trichloroacetic acid (TCA) at zero time. The other reaction mixture incubated at 30°C, was shaken with TCA in the same manner after a 4 hr. incubation. Activity was followed both colorimetrically and spectro-photometrically. The effect of some activators and inhibitors and also that of temperature were investigated as follows: To the supernatant solution, $(NH_4)_2SO_4$ was added up to 0.6 saturation, the solution centrifuged at 7000×g, 10 min., then the resultant precipitate resuspended in a minimal volume of phosphate buffer of pH 7.0. This partially purified preparation was used as the enzyme solution. Reaction mixtures containing 3 ml of enzyme solution, 2 ml of 5% casein, 2 ml of buffer and 2 ml of 0.002 M activator or inhibitor were prepared, and incubated for 2 hrs. at 30°C. Before and after incubation the reaction mixtures were shaken with 11 ml of 30% TCA.

The effect of temperature was tested with neither activators nor inhibitors. The activity determination was carried out spectrophotometrically.

In the colorimetric method, to 1 ml of the TCA filtrate, 5 ml of 0.4 M Na_2CO_3 and 1 ml of Folin reagent were added. After allowing to stand at room temperature for 20 min., the colour development was determined. In the spectrophotometric method, 4 ml aliquots of each filtrate were diluted to 10 ml with distilled water, and their absorption at 280 mμ was determined with a Shimazu quarts-spectrophotometer. Proteolytic activity was expressed as the optical density increment of the incubated solution minus those of the corresponding blanks incubated without either the substrate or the enzyme.

In dealing with black rot sweet potato, attention must be paid to the fact that a considerable amount of polyphenolic compounds accumulate in healthy part next to the infected, and when exposed to air on homogenation of the sample, these compounds are subjected to rapid oxidation to quinones which react with amino acids and perhaps also with proteins, thus consequently resulting in the accumulation of melanin compounds[9]. These groups of compounds produced might more or less exert influence upon the proteolytic enzyme activity itself and the measurements of activity by the 280 mμ absorption. The plant tissues were, therefore, homogenized with cold acetone at −20~−30°C and dried into powder. Such treatments proved very useful, in order to get rid of various contaminating compounds soluble in organic

TABLE I

PROCEDURES OF THE ACETONE POWDER PREPARATIONS AND OF THE EXTRACTION OF ENZYME

Sample 5 g
cold acetone 100 ml
↓
Homogenate 3′
↓
Wash with
cold acetone 100 ml
cold ether 100 ml
↓
Keep in a desiccator
contg. P_2O_5
↓
Acetone Powder
↓
extract with
3-fold volume of
$\frac{M}{15}$ phosphate buffer (pH 7.0)
↓
Centrifuge at 1000×g 15′
↓
ppt. | Supernatant
used as the enzyme solution

9) I. Uritani and M. Miyano, *Nature*, **175**, 812 (1955).

solvents. The procedures of the acetone powder preparation and the extraction of enzyme are summarized in Tab. I. The reaction mixture contained 1.5 ml of enzyme solution, 1.5 ml of 5% casein and 1.5 ml of buffer of pH 7.0. The test solution was incubated for 2 hrs. at 35°C. After incubation, 1.5 ml of 30% TCA was added.

Activity determination was carried out both colorimetrically and spectrophotometrically, as described above. In order to calculate the activity per mg. of protein-N, the amount of nitrogen in the enzyme solution was determined on double specimens by the semimicro Kjeldahl method.

RESULTS

Optimum pH The effect of pH upon the rate of hydrolysis of casein by the crude enzyme preparation is represented in Fig. 1.

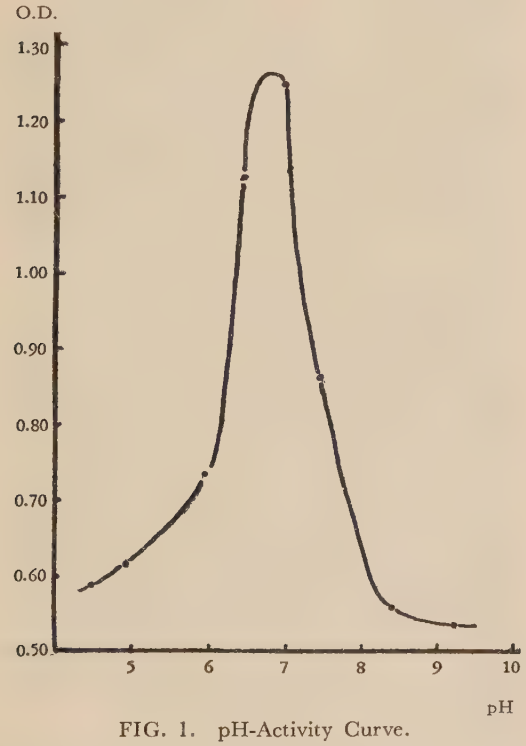

FIG. 1. pH-Activity Curve.

This rate seems to be maximal near the neautral point in phosphate buffer.

Optimum Temperature A maximal proteolytic activity can be observed near 35°C. (Fig. 2).

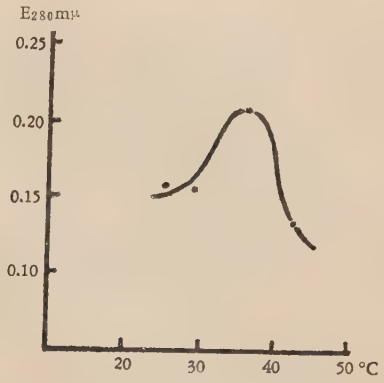

FIG. 2. Temperature Activity Curve.

Effect of Activators and Inhibitors The effect of several activators and inhibitors tested by measuring the degree of digestion, with and without the activators or inhibitors. In view of the experimental results shown in Table II

TABLE II

EFFECT OF ACTIVATORS AND INHIBITORS ON PROTEOLYTIC ACTIVITY

Expt. 1		Expt. 2	
Fraction	E_{280} mμ	Fraction	E_{280} mμ
Crude extr.	0.018	0.6 sat. fr.	0.090
0.6 sat. fract.	0.091	〃 +B. extr.*	0.138
〃 +Cyst-SH	0.063	〃 $+Fe^{+3}$	0.134
〃 +EDTA	0.066	〃 $+Fe^{+2}$	0.080
〃 +KCN	0.053	〃 $+Mg^{+2}$	0.088
〃 +PCMB	0.096	〃 $+Mn^{+2}$	0.092
		〃 $+Cu^{+2}$	0.082
		〃 $+Zn^{+2}$	0.086
		〃 +Citrate	0.050
Final conc. of agents:		0.002 M in Expt. 1. 0.0002 M in Expt. 2.	

* Boiled Extract.

relating that the addition of chelating agents such as ethylenediaminetetraacetate, citrate and potassium cyanide resulted in thirty to forty per cent inhibition, and that *p*-chloromercuribenzoate which is a SH inhibitor was as ineffective as cysteine, which is commonly known as a SH activating agent, it can be considered that the sulfhydryl group may not be responsible for the emergence of proteolytic activity while some kinds of cations are very probable to be effectively involved. Among all the metallic cations

tested, ferric ion was found to cause a 50% acceleration of hydrolysis at a concentration of 0.0002 M. This effectiveness of ferric ion was also ascertained by the Folin method.

The Proteolytic Activity of Black Rot Sweet Potato An individual of sweet potato root was separated into two parts. One half was kept at 25°C. in the raw state (raw). The other half was sliced (2 cm-thick), each of the slices being cut further into two portions of which the one was infected with *Ceratostomella fimbriata* (c.f.) and the other held without infection at 25°C. (control). The control specimen was eatablished to examine the effect of slicing. Representative data of a series of experiments are shown in Fig. 3.

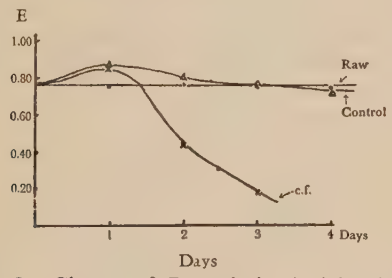

FIG. 3. Change of Proteolytic Activity in Black Rot Sweet Potato.

Activity measured by the Folin method was expressed as O.D. per mg proteine N.

From this it can be seen that the proteolytic activity of black rot sweet potato declines appreciably at the 48 th hr. while in case of both healthy and control specimens, activity remains almost at the same level even after 72 hrs. Such a diminution in proteolytic activity of the diseased tissues was also observed when another variety of sweet potato (Norin No. 10 var.) was tested

DISCUSSION

From the beginning it has been expected that proteolytic enzymes must exist in an active state in black rot sweet potato, since protein synthesis and protein reorganization observed in the metabolically activated tissues will require the activation of a sequence of processes which may supply amino acids and other lower molecular compounds for building bricks and which also must be smoothly pushed forth by the participation of proteolytic enzymes. The results obtained are, however, not in conformity with what we have expected. A more working explanation will be possible, i.e., that the activation of the protein synthesizing system will be evoked on the one hand, while on the other hand activity of the breakdown system gradually becomes retarded, protein synthesis thus being accelerated in a duplicate sense.

It is, however, dangerous to directly assume that the decrease in proteolytic activity signifies something resembling decrease in the turnover rate of protein metabolism. The protoplasm in the sound tissue adjacent to the infected will be activated by the stimulus of fungus penetration, and the proteolytic enzymes may be situated in the active cells in a state that makes them possible to act on the substrates more freely. In the discussion of the turn-over rate, therefore, the state of protoplasm, besides the activity of the enzymes, must be taken into consideration. Nothing should be mentioned on these points until further studies, for instance, the application of isotope techniques supply us more direct evidences. Be that as it may, the fact that the falling- off of proteolytic activity in black rot sweet potato has been demonstrated in vitro, must possess biochemical significance in consideration of the mechanism of the host-parasite interaction, in higher plants.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 4, p. 215~218, 1956]

Terpenoids

Part I. The Structure of Occidentalol, a New Sesquiterpene Alcohol from *Thuja occidentalis* L.

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Received July 6, 1956

A new sesquiterpene alcohol, $C_{15}H_{24}O$, m.p. 95°C, named occidentalol, has been isolated from the essential oil of the wood of *Thuja occidentalis* L. The structure of this occidentalol is most probably represented by formula (I).

It has been reported by one of us[1] (T.N.), that the acidic fraction of the essential oil from the wood of *Thuja occidentalis* L., is composed of α- and β- thujaplicin and that the former is the major constituent of the acidic fraction. This paper deals with the structure of a new crystalline substance isolated from the neutral fraction of the same essential oil.

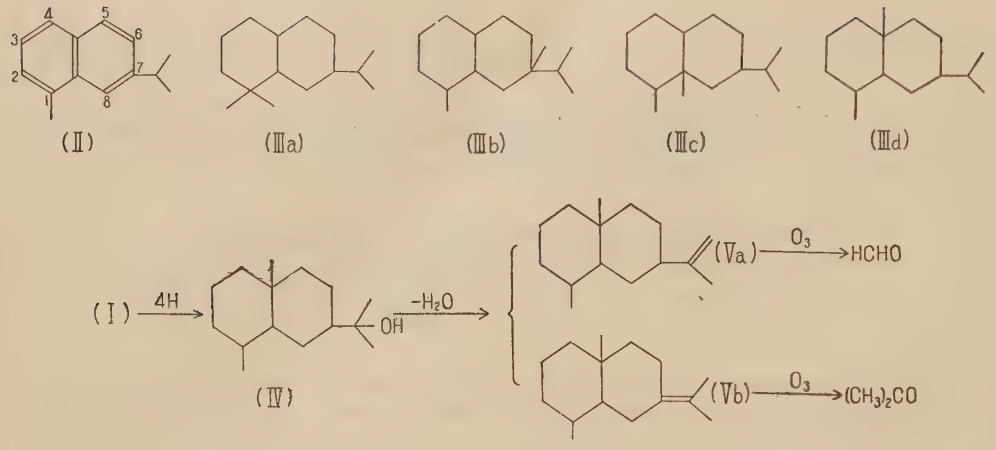
By recrystallization from petroleum ether and sublimation a colorless crystalline substance, for which the name occidentalol is proposed, $C_{15}H_{24}O$, m.p. 95°C, $[\alpha]_D^{24}+361°$, has been isolated from the fraction, b.p. 115–125°C/3 mm, of the neutral oil. Occidentalol is soluble in ether, chloroform, petroleum ether, acetone, alcohol, benzene, insoluble in water, and does not give the ferric chloride reaction. Reddish-brown coloration with tetranitromethane and absorption of bromine prove its unsaturated nature. Neutral potassium permanganate in acetone solution is decolorized.

3,5-Dinitrobenzoate melts at 137°C. The presence of the oxygen atom is recognized as a tertiary alcoholic group because of its non-reactivity with phthalic anhydride in benzene solution and even at the range of 130–140°C, its ease of dehydration with formic acid to a hydrocarbon or mixture of hydrocarbons named occidentalene, $C_{15}H_{22}$, b.p. 86–87°C/2 mm and furthermore its infrared absorption band in the region of 1100–1200 cm^{-1}.

On catalytic hydrogenation with palladium charcoal in alcohol solution, occidentalol gives dihydrooccidentalol $C_{15}H_{26}O$, m.p. 87.5–88.5°C, yellow coloration with tetranitromethane. If hydrogenation is carried out with Adams' catalyst in glacial acetic acid or in a mixture of glacial acetic acid and alcohol, the saturated tetrahydrooccidentalol, m.p. about 80–87°C, is obtained. These facts suggest that occidentalol has two ethylenic linkages possessing different property.

On dehydrogenation with palladium charcoal, eudalene (II) is afforded. This suggests the elimination of a methyl group of an angular or gemalkyl type. The carbon skeleton of occidentalol, therefore, is represented by either (IIIa), (IIIb), (IIIc), or (IIId). Formula (IIIa) is excluded, since the gemmethyl group can not be in position 1, by the reason of the existence of an ethylenic linkage in this position as described later. There are two reasons to deny formula (IIIb), the one is that 1,7-dimethylnaphthalene is not given by dehydrogenation and the other is that the hydrocarbon (Vb), possessing the isopro-

1) T. Nakatsuka, *J. Japan Forest Soc.*, **34**, 248 (1952).



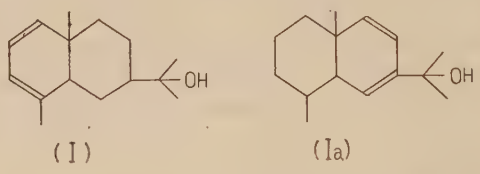
pylidene group at the position 7, is obtained by dehydration of tetrahydrooccidentalol, as described later. Formula (IIIc) is improbable, since the isoprene rule is not applicable. Although rigid chemical proof has not yet been obtained, formula (IIId) is most probable because the isoprene rule can be applied to it and besides sesquiterpene and alcohol of the eudalene type, e.g. selinene and eudesmol, possess this carbon skeleton.

The saturated tetrahydrooccidentalol must be represented by formula (IV), since ozonolysis of tetrahydrooccidentalene (Va, b), derived from the saturated tetrahydrooccidentalol by dehydration with formic acid, gives formaldehyde and acetone.

Occidentalol showed infrared absorption bands at 1590, 845 and 720 cm^{-1}, which are characteristic of a conjugated system, a trisubstituted and di(*cis*)-substituted double bond, respectively. Dihydrooccidentalol has no band near 1600 cm^{-1}, indicating the disappearance of a conjugated system. This is in agreement with the behavior of the double bonds on catalytic hydrogenation. The ultraviolet absorption maximum at 266 mμ, log ε 3.6, is indicative of the presence of a conjugated system placed in one ring.

The structure of occidentalol must be either (I) or (Ia), in order to satisfy the above mentioned nature of the two ethylenic linkages.

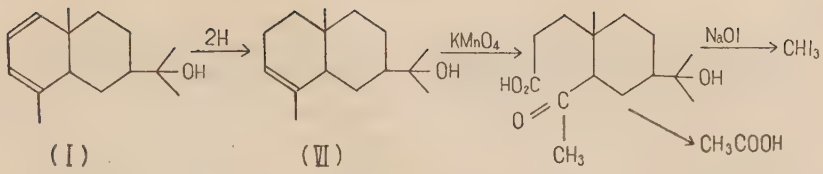
On oxidation of dihydrooccidentalol, pre-



pared by catalytic hydrogenation with palladium charcoal in alcohol solution, with potassium permanganate in acetone solution, the resulting acidic substance, although not isolated in the pure state, gives the iodoform reaction and acetic acid is obtained. This fact is explained and shown in the following scheme, assuming that the structures of occidentalol and dihydrooccidentalol are to be represented by both formulae (I) and (VI), respectively.

When the calculation of the ultraviolet spectra data which L.F. Fieser and M. Fieser[2] demonstrated in the sterol field is extended to bicyclic sesquiterpene, the calculated value for formula (I) is in good agreement with the observed one. Thus, the calculated maximum for occidentalol is: 253 (parent homoannular diene)+15 (3 substituents)=

2) L.F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene", Reinhold Pub. Cor., 1949, p.p. 184-187.



268 mμ (observed, 266 mμ).

Although the yielding of maleic anhydride adduct and reduction with sodium and alcohol have not been attained, occidentalol is most probably represented by formula (I).

Since a very small amount of formaldehyde is obtained by the ozonolysis of occidentalol, the presence of a small quantity of an isomer, having an exocyclic linkage, in occidentalol is suggested.

In addition to occidentalol, a sesquiterpene alcohol, $C_{15}H_{24}O$, m.p. 69–70°C, $[\alpha]_D^{25}+163.7°$, 3,5-dinitrobenzoate m.p. 166–167°C, has been isolated from the higher fraction, b.p. 135–145°C/3 mm of the same oil. Experiments on its structure are now in progress.

EXPERIMENTAL

Isolation of Occidentalol. The neutral substances (460 g) of the essential oil of *Thuja occidentalis* L. were distilled twice, so as to give results as listed in the Table.

TABLE

b.p. °C/3 mm	Yield. g	Yield. %	Appearance.
(1) 115–125	237	51.4	slightly colored solid
(2) 125–135	86	18.6	orange colored viscous liquid
(3) 135–145	43	9.3	〃
(4) 145–157	61	13.5	yellowish solid
(5) loss and residue	33	7.2	--

A mixture of fractions (1) and (2) was re-distilled and the fraction, b.p. 115–125°C/3 mm, was recrystallized from petroleum ether, followed by sublimation. The compound was colorless and melted at 95°C, $[\alpha]_D^{24}+361°$ (c=2.440, in chloroform).

Analysis. Found: C, 81.58; H, 10.33. M.W. 222 (Rast method). Calcd. for $C_{15}H_{24}O$: C, 81.76; H, 10.98%. M.W. 220. The ultraviolet absorption spectrum: λ_{max}^{MeOH} 266 mμ, log ε 3.6.

Esterification. A mixture of occidentalol (0.5 g) and 3,5-dinitrobenzoyl chloride (0.8 g) in pyridine was heated for 5 min. and the reaction product was poured into water. The precipitate separated, was washed with 5% sodium carbonate solution and water, and then recrystallized from alcohol, m.p. 137°C.

Analysis. Found: C, 63.34; 6.10; N, 6.91. Calcd. for $C_{22}H_{26}O_6N_2$: C, 63.75; H, 6.32; N, 6.76%.

Occidentalol did not react with phthalic anhydride in benzene solution and even at the range of 130–140°C.

Hydrogenation of Occidentalol. Occidentalol (64.8 mg), palladium charcoal (10 mg) and absolute alcohol (30 cc) were shaken together in hydrogen until absorption (6.71 ml; the theoretical amount for one double bond, 6.60 ml) was completed. Filtration, evaporation and crystallization from aqueous alcohol, followed by sublimation gave dihydrooccidentalol, m.p. 87.5–88.5°C, $[\alpha]_D^{24}+59.2°$ (c=2.990, in chloroform). It gave a yellowish coloration with tetranitromethane.

Analysis. Found: C, 80.63; H, 11.51. Calcd. for $C_{15}H_{26}O$: C, 81.02; H, 11.79%.

Occidentalol (81.0 mg), platinic oxide (15 mg) and glacial acetic acid (30 cc) were shaken together under hydrogen until absorption was ceased (40 min.); the uptake was 17.30 ml (the theoretical amount for two double bonds, 16.70 ml). Filtration, evaporation in vacuo and addition of water gave tetrahydrooccidentalol, which was not colored with tetranitromethane. Although recrystallization and sublimation were repeated, the product was heterogeneous, showing no sharp melting point, 80–87°C.

Dehydration of Occidentalol. A mixture of occidentalol (3 g) and about 90% formic acid (15 cc) was heated on a water bath for 30 min. The upper oily layer was washed with sodium carbonate solution and sodium chloride solution. On distillation, a colorless liquid, b.p. 93–98°C/3 mm was obtained. Repeated distillation over metalic sodium gave a colorless liquid (1.2 g), b.p. 86–87°C/2 mm, n_D^{20} 1.5123, d_4^{20} 0.9254, M.R. Obsd: 65.62. Calcd. for $C_{15}H_{22}F_3$: 64.67. It became sticky on standing in the air.

Analysis. Found: C, 88.69; H, 11.07. Calcd. for $C_{15}H_{22}$: C, 89.04; H, 10.96%.

Dehydrogenation. A mixture of occidentalol (5 g) and palladium charcoal (0.5 g) was heated for 6 hr. at 260–270°C. The ether-soluble product was distilled to give the following fractions, b.p. °C/2 mm (1) 87–92, (2) 92–100, (3) 100–150. The principal fraction (1) (2 g) was distilled twice over metalic sodium, b.p. 264–267°C, d_4^{19} 0.946, n_D^{19} 1.5286. M.R. Obsd: 60.07. Calcd. for $C_{14}H_{16}F_5$: 60.08. This hydrocarbon afforded picrate, m.p. 93–94°C, and trinitrobenzene derivative, m.p. 112–113°C. The melting points of these two substances were unaltered in mixture with those of synthesized eudalene. The picrate was passed through a column of alumina and eluted with petroleum ether to afford regenerated liquid, which had characteristic ultraviolet absorption bands of naphthalene at 280, 320 and 322 mμ (log ε 3.7, 2.4 and 2.0, respectively).

Ozonolysis of Occidentalol. Occidentalol (2 g) in chloroform (30 cc) at −15°C was treated with ozone in excess, and the yellow viscid liquid obtained by evaporation of the solvent under reduced pressure was treated with water, and then steam-distilled. The distillate gave a small amount of formaldehyde-dimedone compound, m.p. and mixed m.p. 189–190°C.

Dehydration of Tetrahydrooccidentalol. Tetrahydrooccidentalol (4 g) was heated with 90% formic acid (20 cc) on a water bath for 30 min. The product was distilled in vacuo over metalic sodium, b.p. 90–93°C/4 mm, n_D^{21} 1.4876, d_4^{21} 0.9008, M.R. Obsd: 66.0. Calcd. for $C_{15}H_{26}F_1$: 66.6, $[\alpha]_D^{26}$ −38.6° (c=4.472, in chloroform).

Analysis. Found: C, 87.80; H, 12.42. Calcd. for $C_{15}H_{26}$: C, 87.30; H, 12.70%.

Ozonolysis of Tetrahydrooccidentalene. Tetrahydrooccidentalene (0.2 g) in chloroform was treated with ozone for 1.5 hr. until the solution gave a negative tetranitromethane test. The ozonide obtained by evaporation of the solvent under reduced pressure was treated with water, first at room temperature and then on a boiling water bath for two hours. The aqueous solution was steam-distilled, and the distillate, after shaking with ether, was treated with a solution of dimedone in methanol. After standing for 24 hr. at 0°C, the crystalline formaldehyde-dimedone compound was collected and recrystallized from methanol, m.p. and mixed m.p. 189–190°C.

Tetrahydrooccidentalene (2 g) in chloroform was ozonized at −20°C for 4 hr., no coloration with tetranitromethane. After the decomposition of ozonide by water, it was steam-distilled. The first distillate gave 2,4-dinitrophenylhydrazone, m.p. 124°C (from methanol), identified as acetone derivative by mixed m.p. The second portion of steam-distillation gave a formaldehyde-dimedone compound, m.p. and mixed m.p. 189–190°C, by a similar procedure as described above.

Oxidation of Dihydrooccidentalol with Potassium Permanganate. To a solution of dihydrooccidentalol (2 g) in aqueous acetone (30 cc), powdered potassium permanganate (10 g) was added in portions with stirring at room temperature. After filtration and evaporation of acetone, the unattacked dihydro compound was separated. The filtrate was shaken with ether. The aqueous layer was acidified with dilute sulfuric acid and shaken with ether. After washing the ethereal solution with water to remove the water soluble acidic fraction, evaporation of the ether afforded a yellow oily substance (0.5 g). To a solution of the acidic substance in 5% sodium hydroxide solution, iodo-potassiumiodide solution was added. Yellow powder thus separated, having an odor of iodoform, was recrystallized from aqueous alcohol, m.p. 119–120°C. The washing water containing the water soluble acid, was neutralized and evaporated to dryness to obtain acid salt (0.3 g). *p*-Toluidide, prepared in the usual way, had m.p. 146–147°C undepressed by mixing with the *p*-toluidide of acetic acid.

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Studies on the Abnormal Metabolism in Animals Poisoned with Sodium Fluoroacetate

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The changes of metabolisms in the calf, dog and rat following administeration of fluoroacetate were studied. It was found that citrate is accumulated in the tissues, and increased amounts of acetone bodies are excreted in the urine of the animals which received fluoroacetate, and their urinary nitrogen also increases. All these abnormal accumulations and excretions of the rat after injection of fluoroacetate diminish within 24 hrs., suggesting the decrease of the toxity or the excretion of the poison substance.

Significant amounts of organic fluorine compounds were detected in various tissues of fluoroacetate-treated calves.

The lethal dose limit of fluoroacetate for the calf could not be determined. As for the dog, 0.02 mg/kilo of fluoroacetate seemed to be fatal.

Fluoroacetic acid is known to exhibit an inhibiting effect on citrate oxidation in animals, thus resulting in the accumulation of citrate in various tissues[1-7]. This inhibition has been proved to be due to the competitive inhibition of aconitase by flouorocitrate[8-12], which is enzymatically synthesized by the condensation of fluoroacetate and oxaloacetate, mediated with CoA as the carrier of fluoroacetyl moiety, possibly, in the same manner as the first step of acetate metabolism *via* the TCA cycle[13,14].

The possible changes of the metabolism of amino acids, such as glutamate and aspartate, which are known to enter the TCA cycle after deamination or transamination, have been investigated. Significant losses of these amino acids and glutamine[15] with the increase in the amounts of ammonium ion[7] have been demonstrated in some tissues of animals after administeration of fluoroacetic acid.

The blocking of the TCA cycle by fluorocitrate also results in changes of the main metabolic pathway of fats[6,17] and carbohydrates[16], especially in the CoA-relating step, and lack of oxaloacetic acid would promote the condensation of the acetyl radicals of acetyl CoA, followed by the increase in the amounts of acetone bodies in tissues and urine.

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TABLE I
THE RECORDS OF THE DOSAGE OF SODIUM MONOFLUOROACETATE (1080) ON THREE HEALTHY CALVES

Calf No.	Age	Sex	Weight	1080 administered		Survival time
				Total	per weight	
	months		kg	mg	mg/kg	hr.
1	12	♀	160	480	30	2
2	6	♂	100	50	0.5	8
3	7	♂	150	15+30*	0.1+0.2*	38

* Received 15 mg of 1080, and additional 30 mg on the following morning.

This paper reports the accumulation of citrate in tissues, increase of acetone bodies in blood and urine, and excretion of nitrogen in urine of calves and rats after the administeration of fluoroacetate. The lethal dose limit of fluoroacetate for calves and the distribution of fluoroacetate in several tissues were also studied.

EXPERIMENTAL

Aqueous solution of sodium monofluoroacetate "1080" was administered orally to three healthy calves (from 6 to 12 months old) using the gastric tube, in the morning previous to feeding. The tissues were removed immediately after death, and then subjected to analyses. To the dogs, the poison substance was administered in the almost the same way as the calves. To the rats, 1.5 mg of fluoroacetic acid (neutralized with NaOH) per kilo, was administered by intraperitoneal injections. Analyses were carried out also immediately after sacrifice of the rats.

The estimation of citrate was carried out according to the method of Buffa and Peters[1]. Acetone bodies were determined by the method of Greenberg and Lester[19], and nitrogen by the Kjeldahl method.

Monofluoroacetate was estimated by the method of Ramsey and Clifford[18], based upon the estimation of total fluorine in the organic acid fraction which was completely freed from the inorganic fluorine partition chromatographically.

RESULTS AND DISCUSSION

Determination of the Lethal Dose Limit. The varieties of the pharmacological actions of fluoroacetate on the different species and also on different strains of the same species were explicitly shown in the comprehensive review of Chenoweth[20]. But the lethal dose limit of the poison for the calves was not given in that review. The data described in Table I, show that the increased amounts of poison, "1080", shortened the survival time of the calves. Though the lethal dose limit for calves could not be determined directly from such scanty data, however, 0.2–0.3 mg per kilo of the doses to calves seems to be fatal. On the other hand, the healthy dogs receiving 0.02 mg per kilo died with convulsion, this fact coinciding with the observations by Suzuki[21], and Frick and Boebel[22]. This lethal dose limit, 0.02 mg per kilo, is extremely low in comparison with the value reported by Peters (0.06 mg per kilo)[8].

A possible explanation of the lower value of the lethal dose limit of fluoroacetate obtained here, is that the poisoning effect of fluoroacetic acid might be closely dependent of health and nutrition conditions of animals, because its action, differing from that of another animal poison, i.e., potassium cyanide and mercuric chloride, is directly related to the early steps of the TCA cycle — the main pathway of energy metabolism.

Distribution of Sodium Fluoroacetate in Calf. The distribution of monofluoroacetate in the poisoned calves is shown in Table II. The amounts of fluoroacetate in the organs,

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TABLE II

DISTRIBUTION OF SODIUM FLUOROACETATE IN THE TISSUES OF THE CALVES ABOVE (Table I.)

Calf No.		1			2			3	
Total does (mg)		480			50			45	
Organ	Wt.	Amount of 1080		Wt.	Amount of 1080		Wt.	Amounts of 1080	
		Recovery	Concn.		Recovery	Concn.		Recovery	Concn.
	g	mg	ppm	g	mg	ppm	g	mg	ppm
Kidney	470	21.4	45.5	330	2.6	7.8	510	0.7	1.3
Heart	800	8.7	10.9	620	6.0	9.6	740	3.8	5.1
Liver	2350	204.9	87.2	1860	7.1	3.8	2250	1.6	0.7
Cerebrum	350	—	—	356	—	—	380	1.0	2.7
Urine	525 (cc.)	2.7	5.1	900 (cc.)	1.2	1.3	7395 (cc.)	2.2	0.3

TABLE III

RELATION BETWEEN THE DOSES OF SODIUM FLUOROACETATE AND THE CITRATE ACCUMULATION IN THE TISSUES OF THE CALVES

Calf No.	Dose	Survival time	Amounts of citric acid in			
			Kidney	Heart	Cerebrum	Diaphragm
	mg/kg	hr.	μg/g	μg/g	μg/g	μg/g
1	3.0	2	84	45	46	54
2	0.5	8	148	154	53	68
3	0.1+0.2	38	192	488	26	180
Control	0	—	25	14	36	19

TABLE IV

RELATION BETWEEN THE DOSES OF SODIUM FLUOROACETATE AND THE CITRATE ACCUMULATION IN THE TISSUES OF THE DOGS

Calf. No.	Dose	Survival time	Amounts of citric acid in			
			Kidney	Heart	Cerebrum	Diaphragm
	mg/kg	hr.	μg/g	μg/g	μg/g	μg/g
1	0.087	7	385	143	270	—
2	0.043	23	1215	—	223	608
Control	0	—	61	33	47	47

especially in the liver and kidney, varied considerably in accordance with the doses, but was approximately constant in the heart. These values in the heart are in good agreement with that reported by Ramsey and Clifford[23], and Peters and Buffa[8].

With the findings of the citrate accumulation in the tissues as shown in the Table III and IV and that of fluoroacetate in the heart of the fluoroacetate-treated calves and cattles, it became possible to establish a method for the decision of the fluoroacetic acid-poisoning to calves and cattles.

23) E.C. Hagan, L.L. Ramsey and G. Woodard, *J. Pharm. Exptl. Therap.*, **99**, 432 (1950).

Excretion of Nitrogen in Urine. The increase of nitrogen in the urine of the fluoroacetate-treated calves shown in Fig. 1, suggest alteration on the metabolism of amino acids or proteins by blocking of the TCA cycle.

The Increase of Acetone Bodies in Blood and Urine. The increase of the acetone body excretion by calves and rats after administration of fluoroacetate are shown in Figs. 1 and 2, respectively. These facts may be due to the supression of the incorporation of acetyl CoA into the TCA cycle, caused by the decrease of the TCA cycle acids owing to the inhibition of aconitase by fluoroacetic acid.

As shown above, the amounts of citrate in

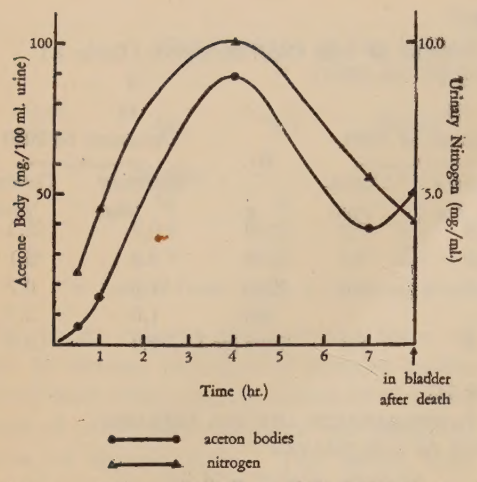

FIG. 1. Effect of Sodium Fluoroacetate on the Urinary Excretion of Acetone Bodies and Nitrogen by Calf after the Lethal Dosage of 0.5 mg of Fluoroacetate per kilo.

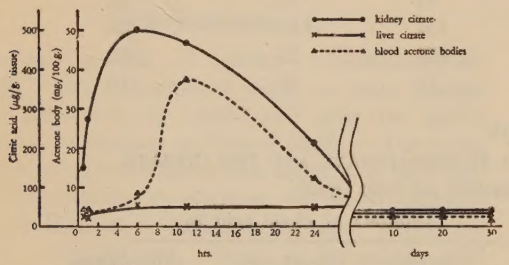

FIG. 2. Effect of Sodium Fluoroacetate on the Acetone Body Excretion and Citrate Accumulation in the Tissues of the Rat after the Intraperitoneal Injection of Non-lethal Dose of the Poison. (1.5 mg/kilo)

tissues, and of acetone bodies in blood and urine, increase toward the maximum levels and then decrease with the elapse of time to normal levels. These facts suggest that the animal body is capable of the breakdown or another detoxication of the poison, followed by excretion or accumulation in those possible nonor less toxic forms[13,14,24,25].

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ADDENDUM

By means of the analyses of citrate and fluoroacetate in the tissues, the authors have somewhat enlightened the problem of violent death of the calves and cattles, at Yodo Basin, Osaka Prefecture. (To dissolve the problem, i.e., to find out the cause of the death of these animals, many strenuous efforts have been expended by other laboratories extending over the last three years.)

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Journal of the Agricultural Chemical Society of Japan.

(Printed in Japanese)
Vol. 30, No. 8 (August, 1956)

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